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(54) Title: MEMBRANE EXPRESSION OF HETEROLOGOUS GENES

(57) Abstract

The invention relates to pharmaceutical compositions and methods of producing bacterial host surface-expressed heterologous polypeptides useful in the preparation of vaccines, and particularly oral vaccines for prophylaxis of diseases associated with cholera, human immunodeficiency virus, influenza virus, and rickettsial infections.

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DESCRIPTION

MEMBRANE EXPRESSION OF HETEROLOGOUS GENES

BACKGROUND OF THE INVENTION

Field of the Invention

The invention relates generally to the field of vaccine development, and particularly to pharmaceutical compositions comprising oral vaccines effective against bacterial and viral diseases such as cholera, rickettsia, influenza and human immunodeficiency virus. In certain aspects, the invention relates to methods of treating these diseases through oral administration of attenuated bacterial strains comprising surface-expressed heterologous polypeptide immunogens.

Description of Related Art

Recombinant gene technology has been extensively investigated in the context of expression of foreign proteins from recombinant host cells, typically bacterial host cells. Such expression is desirable for producing high value proteins, immunogenic polypeptides, and in obtaining hybrid proteins that are otherwise difficult to synthesize.

Isolated epitopes of known antigens of eukaryotic, viral and prokaryotic pathogens are recognized as potentially useful in vaccine development. Preparation of protective vaccines from transformed host cells has been attempted. Oral vaccines have stimulated interest because of the ease of administration and, more importantly, in some instances the unsatisfactory protection afforded from parenteral injection.

While vaccination against cholera has been possible, the effect has been short-term, necessitating the development of longer-term therapies. Sanchez *et al.* (1990) have proposed a possible approach to the problem through the development of an oral vaccine that would presumably stimulate mucosal intestinal immunity more efficiently. However, no oral vaccine against cholera has been developed which is completely effective.

1. Cholera Toxin

Cholera toxin (CT) is an exotoxin produced by *V. cholerae* and is composed of one A subunit (CTA) and five B subunits (CTB) (Holmgren, 1981). The A subunit is a 27 kDa protein which is responsible for enterotoxigenic activity, mediates the ADP-ribosylation of adenylate cyclase (Field, 1971), and induces the release of eicosanoids (Peterson and Ochoa, 1989). The B subunit is a homopentamer consisting of 11.6 kDa subunits (Holmgren, 1981). The primary amino acid structure of CTB (Kurosky *et al.*, 1977) and the complete sequence of the *ctxB* gene (Mekalanos *et al.*, 1983) have been determined. The B subunit functions to bring the enzymatic A subunit in contact with the cell through high affinity binding to GM1 ganglioside which is present on the cell surface (Holmgren, 1981). GM1 ganglioside is widely distributed in the cell membrane of mammalian cells (Holmgren, 1981). Hence, possibly through the ubiquitous nature of its receptor, CT can modulate the activity of a variety of cells. These include not only those directly involved in the cholera diarrheal secretory process, but also cells of the immune system, including lymphocytes and antigen-presenting cells (APC) (Holmgren and Lindholm, 1976). For many of these activities, CTB shares the same properties with the holotoxin (Elson, 1989).

The cholera toxin A and B subunit proteins are tandemly arranged as a single transcriptional unit (Mekalanos *et al.*, 1983). Oxidation of B subunits during export from the cytosol is a prerequisite for pentamerization (Hardy *et al.*, 1988). The B subunit enters the periplasm as a monomer and is assembled into oligomers prior to secretion (Hirst and Holmgren, 1987). Thus, CTB is present in multiple molecular forms in bacterial cells.

2. Host Immune Response to Cholera Toxin

The host immune response to CT has been extensively studied. It is generally accepted that protective immunity against cholera is dependent on the stimulation of the immune system of the gut. Secretory IgA production (sIgA) in response to locally encountered antigens (Svennerholm *et al.*, 1984; Hanson and Brandtzaeg, 1986; Holmgren and Lycke, 1986) is believed to play a major role. Both antibacterial and antitoxic mucosal immunity are observed after natural infection (Holmgren and Svennerholm, 1983) and are associated with protective states. Neutralization of CT by CTB-specific antibodies effectively abrogates CT activity in animal models (Holmgren, 1981; Svennerholm *et al.*, 1984). Antibodies raised to denatured CTB or toxoid are also effective at neutralizing CT activity (Markel *et al.*, 1979).

Local intestinal immune responses are thought to be initiated by antigen uptake mechanisms of specialized epithelial M cells (Pierce and Koster, 1981; Cebra *et al.*, 1976; Husband and Gowens, 1978). For *Salmonella*, penetration of the epithelial lining of the gut, and residence in the lamina propria may also initiate the immune response. Antigens are processed by macrophages, and then presented to immunocompetent T and B cells.

There is evidence for the activation of antigen-specific helper T cells and B cells from Peyers patches (PP) (Elson and Ealding, 1984; Husband and Gowens, 1978; Fuhrman and Cebra, 1981). Stimulated-B cells and possibly T cells migrate to the mesenteric lymph nodes (MLN). Cells are then distributed to different lymphoid tissues and all mucosal sites. A population of committed cells home to the intestinal mucosa, where development to differentiated effector cells occurs. Thus, intestinal immune responses can produce a systemic response and antigen-specific lymphoid cells at all mucosal sites.

There is some indication that the optimal means of stimulating the secretory immune system of the gut is by locally applying the immunogen. Evidence exists which indicates that live oral avirulent *Salmonella* vaccines may be effective in priming the local immune system; however, no studies have shown such priming would be useful in generating an immune response against other unrelated pathogens. Thus the development of oral vaccines which utilize *Salmonella*-induced immune system priming against such unrelated pathogens would constitute a major breakthrough in the field of oral vaccine development.

3. *Salmonella* as a Candidate for Oral Live Vaccines

Salmonella strains have been studied as particularly attractive candidates for producing oral live vaccines. Attenuated strains have elicited immune responses in several animal species (Strugnell *et al.*, 1990) and apparently can be highly immunogenic in the host. Humoral antibody responses as well as local secretory antibody and cellular immune responses have been observed after oral intake (Dougan *et al.*, 1986). Attenuated mutants have been identified via screening procedures such as Tn*phoA* insertions, that permit elimination of

mutations in nonsecreted proteins (Miller *et al.*, 1989). However, Tn*phoA* methods only indicate assessment of integration of the transposon into a gene for a secreted or cytoplasmic protein.

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Surprisingly, little is currently known as to the subcellular placement of a recombinant antigen in an avirulent *Salmonella* strain on the nature of the immune response which is elicited. In this regard, antigen placement may have a profound effect on the quantitative and qualitative aspects of the immune response directed to a recombinant antigen. For example, a recombinant circumsporozoite (CS) antigen expressed cytosolically in *Salmonella* has yielded high levels of cell-mediated immunity with virtually no detectable humoral response (Sadoff *et al.*, 1988). In contrast, the immunodominant epitope of the CS antigen expressed in a recombinant

flagellin protein on the *Salmonella* surface produces an antibody response (Majarian *et al.*, 1989). Thus, the location of an antigen, inside the bacterial cell versus on the surface of the bacterium, appears to influence the nature of the immune response.

4. Protein Expression Systems in *Salmonella* sp.

Protein expression systems have been developed from *Salmonella* strains. A cloning vector useful for integrating DNA into the *Salmonella* chromosomal *aroC* gene has been used to express heterologous antigens such as tetanus toxin fragment C and *Treponema pallidum* lipoprotein (Strugnell *et al.*, 1990). In some cases, heterologous polypeptide gene products orally administered have elicited a serum antibody response; for example, the heterologous cholera toxin B subunit protein expressed from a recombinant *Yersinia enterocolitica* strain (Sory and Cornelis, 1990). Unfortunately, while antibodies were detected in sera of inoculated mice, the response was variable and was directed toward polymeric forms of cholera toxin B.

It is recognized that antigenic proteins may not produce a strong immunogenic response, possibly because of masking of epitopic regions. Likewise, potential antigens expressed as heterologous proteins from recombinant host cells often lack antibody reactivity (Sanchez *et al.*, 1990). It is believed that even minor modifications of a given antigenic region may lead to complete sequestering of an epitope. While surface expressed epitopes of bacteria may be expected generally to elicit the greatest humoral response, factors controlling surface expression of heterologous proteins have not been defined and there is no way to assure that a fusion protein will localize to a host cell membrane surface.

Despite disappointing results in effecting immunity, the use of attenuated *Salmonella* strains to express heterologous antigens and stimulate GALT is being extensively investigated. In some studies, detectable levels of specific mucosal and serum antibodies to the heterologously expressed antigen have been observed (Black *et al.*, 1987; Curtiss *et al.*, 1987; Dougan *et al.*, 1986; Dougan *et al.*, 1987). However results with most antigens have generally been variable. Humoral response to *Salmonella*-expressed *E. coli* K88 fimbriae (Dougan *et al.*, 1986) and β-galactosidase (Brown *et al.*, 1987), as well as humoral and secretory responses to the *Streptococcus* mutants GFT A polypeptide (Curtiss *et al.*, 1986) and *Shigella* type and group antigens (Black *et al.*, 1987) have been reported. Thus, while these studies have established the utility of *Salmonella* expression of heterologous antigens in generating a specific immune response, and while significant specific mucosal and serum responses to some antigens have been observed, the nature and extent of immune responses to other expressed antigens have been variable. The export of heterologous antigens to the cell surface may enhance their interaction with the immune system (Fields, 1986) possibly through enhancement of T cell-independent mechanisms.

Investigators have expressed heterologous epitopes as inserts in surface-expressed *Salmonella* flagellin or *lamB* protein. Flagellin with inserts specifying a CTB subunit epitope (Jacob *et al.*, 1983; Newton *et al.*, 1989), a hepatitis B surface polypeptide epitope (Wu *et al.*, 1989), and M protein epitopes (Newton *et al.*, 1990) have also induced a humoral response. *Salmonella*-expressed *lamB* hybrid genes expressing VPI epitopes of polio virus (Charbit *et al.*, 1988), pre-S2 of hepatitis B (Charbit *et al.*, 1987), and HIV epitopes (O'Callaghan

et al., 1990) yielded similar results. Significant limitation of these systems include: 1) the relatively small number of epitopes which can be inserted into these gene hybrids; and 2) the relatively small size of DNA insert possible in the flagellin fusions.

5. Deficiencies of the Prior Art

The relatively poor and vague understanding of the immune response to the placement, conformation and processing of bacterial-based antigens which lead to secretory, humoral or cell-mediated immune responses, has provided little information concerning the issue of subcellular placement of antigens and their corresponding effects on the immune response. Previously, evaluating the antigenicity of the same molecule in various subcellular locations has been technically difficult. In spite of limited knowledge in this area, recombinant-based antigen placement vectors have been developed (Curtiss *et al.*, 1989) which "place" recombinant antigens in the periplasm, membranes, or within surface structures (i.e. flagella), or which secrete these molecules.

Although studies have demonstrated some aspects of immune responsiveness to a few antigens, no studies have addressed various subcellular placements of the same antigen on the immune response. There is clearly a need to develop effective systems to elicit antibody response and in particular to provide methods of exporting heterologous polypeptides to the surface of appropriate host cells.

SUMMARY OF THE INVENTION

The present invention overcomes the deficiencies in the prior art by providing novel compositions and methods for the production of oral vaccines comprising *Salmonella* surface-expressed heterologous immunogenic polypeptides.

The invention also includes nucleic acid segments useful for preparing expression vectors which encode these heterologous polypeptides. Such vectors are suitable for transforming host cells to produce heterologous polypeptides exported to selected areas of the host cell.

The nucleic acid segments of the present invention encode amino acid sequences associated with targeting of fused heterologous polypeptides to specific areas of a transformed host cell. It has been found for example that nucleic acid segments defined by SEQ ID NO:1 encode a polypeptide product that when fused to a heterologous polypeptide will direct that polypeptide to the outer membrane of a bacterial cell. By heterologous polypeptide is meant any polypeptide other than those normally associated with the peptide encoded by SEQ ID NO:1. It is of course understood that such localizing capabilities are realized under conditions when the exportation polypeptide is incorporated into a suitable expression vector and an appropriate cell host is transformed with that vector. A preferred embodiment of the DNA segment defined by SEQ ID NO:1 encodes a 50 kDa polypeptide with surface-directing properties.

The present invention also includes nucleic acid segments encoding amino acid sequences associated with the transport of heterologous polypeptides to a bacterial inner membrane periplasmic space. Particular embodiments of the pres nt invention includes the nucleic acid

sequence of SEQ ID NO:2. This sequence encodes a 63 kDa preferred inner membrane/periplasmic space-directing polypeptide. This preferred embodiment includes gene sequences encoding part of the APase gene, however, other heterologous genes may be used in place of APase.

While particular nucleic acid sequences have been defined, it is nevertheless contemplated that nucleic acid sequences will be found to vary. It is expected that analogous sequences with similar functions may be found in other gram-negative bacteria such as *E. coli* or other bacteria having homology with *Salmonella* such as those bacteria with 40% or greater homology.

More distantly related or unrelated sequences which provide an analogous function could be found in gram positive bacteria such as the enterococci, *Listeria monocytogenes*, *Corynebacterium* sp., streptococcus species, and anaerobic bacteria such as *Clostridium* species. Also, *Neisseria* sp., mycoplasma species and *Vibrio* species and *Yersinia* species especially *Y. enterocolitica* are Gram-negative bacteria in which sequences of similar function could be found.

It is also anticipated that analogous sequences could be found in yeast such as *Saccharomyces* and others which could be used for the same purposes. Eucaryotic cells such as those which can be propagated in tissue culture and analogous export-directing sequences are anticipated to be able to perform similar functions. Insect cells such as those commonly used to propagate Baculovirus and their recombinant derivatives would also imagined to function in these methods.

In certain particular embodiments, the invention concerns expression vectors that are constructed to include any of the DNA segments herein disclosed. Such

DNA may be fused directly with a gene of interest and used in an expression system to produce heterologous polypeptides as hybridization probes for, *e.g.*, identifying related sequences, as primers, or even as building blocks for the construction of mutant or variant sequences. A particularly useful application of the DNA segments of this invention is to achieve directed expression of heterologous polypeptides. Depending on the DNA segment selected, polypeptides will be expressed on the periplasmic face of the inner membrane, associated with the outer membrane of the host cell, or on the surface of the outer membrane of the host cell.

In a particular embodiment, pZIP plasmids have been constructed. Depending on the plasmid selected, fusion polypeptides are exported to the periplasmic face of the cytoplasmic membrane or to the outer membrane of the host cell. In a preferred embodiment, pZIP-OUT directs the export of fusion polypeptides to the outer membrane at the external surface of a Gram-negative host cell. pZIP-OUT is a bipartite fusion including a *Salmonella typhimurium* DNA segment capable of exporting the fusion product to the external membrane of a Gram-negative cell. The second part of the chimeric gene is an *Escherichia coli* gene encoding APase (*phoA*). This *phoA* reporter gene segment lacks both regulatory and signal sequences, and the activity of the PhoA fusion polypeptide *in vitro* and *in situ* is readily monitored through the use of chromogenic substrates. A variety of DNAs or DNA segments may be inserted into the *phoA* gene at suitable restriction sites to create in-frame, tripartite fusion.

Yet another preferred embodiment is pZIP-IN. This plasmid directs the export of polypeptides to the inner membrane periplasmic space or the surface of the outer membrane. The construction of the plasmid is basically bipartite. Part of the APase gene lacking signal and

expression sequences is fused with a DNA sequence that contains an exportation sequence capable of directing its fusion polypeptide to the inner membrane/ periplasmic space. There are several restriction sites in the *phoA* gene segment into which foreign DNA or fragments of DNA may be inserted.

Other components of either of these plasmids may include, in addition to the export specifying sequences, resistance genes such as ampicillin or tetracycline resistance genes. Additionally an *E. coli phoA* gene may be fused in frame with expression directing DNA sequences, such as that used to construct the pZIP-IN and pZIP-OUT plasmids. pZIP-IN additionally encodes a kanamycin resistance gene. An advantage of using the *phoA* fusion is that there are various restriction sites within the *phoA* gene facilitating the fusion of heterologous gene sequences in frame with the export specifying sequences.

Expression vectors may also include a gene encoding a detectable polypeptide (a "reporter" gene). Typical examples of polypeptides or reporter genes encoding detectable polypeptides include those for *E. coli* β-lactamase and APase. Reporter genes may be conveniently fused downstream of the disclosed nucleic acid sequences with or without other DNA fragments/segments. Moreover, restriction sites in the gene sequence of the detectable polypeptide may be used for insertion of a desired DNA fragment(s).

Recombinant vectors such as those described are particularly preferred for transforming bacterial host cells. Also it is imagined that recombinant vectors may be employed in gram positive bacteria such as the enterococci, and other streptococcal species, and in BCG or other *Mycobacterium bovis* or other *Mycobacterium*

species derived for vaccine delivery. Several types of bacterial host cells may be employed, most preferred being Gram-negative cells such as *E. coli*, *Salmonella* and the like.

Several avirulent *Salmonella* strains are contemplated as useful in the development of the oral vaccines of the present invention. These orally administered live attenuated vaccines induce specific cell-mediated responses (CMI) and elicit secretory IgA (sIgA) (Sadoff *et al.*, 1988; Poirer *et al.*, 1988; Clements, 1989; Brown *et al.*, 1987. sIgA is especially important because of its effectiveness at mucosal surfaces, sIgA production and CMI responses are mediated through the interaction of antigens with lymphoid cells present in gut associated lymphoid tissue (GALT). Stimulation of GALT can lead to effective cell and humoral defense at all mucosal surfaces and provide systemic protection.

Recent investigations have shown superior mucosal humoral immunity and cellular immunity in animals receiving oral compared to parenteral *Salmonella* immunization (Tagliabue, 1989). Thus, *Salmonella* carrier strains have a demonstrated potential in targeting specific antigens to GALT and in establishing immune states.

A number of avirulent and virulence-attenuated *Salmonella* are contemplated to be useful in the methods disclosed herein. Plasmidless variants, auxotrophic mutants (*aroA*, *pur*), regulatory mutants (*cya/crp*), strains altered in carbohydrate utilization (*galE*) and others (*phoP*) have been investigated as potential carrier strains (Curtiss *et al.*, 1989). These strains can colonize and invade the gut epithelium, persist in tissue, and consistently evoke serum and mucosal antibody

responses with minimal side effects when compared to parental *Salmonella* vaccines (Tagliabue, 1989).

Several virulence-attenuated *Salmonella* strains (Black *et al.*, 1987; Curtiss *et al.*, 1989; Dougan *et al.*, 1986; Dougan *et al.*, 1987; Dougan *et al.*, 1987) may also be useful as host cells in the practice of the present invention. A successful parenteral typhoid vaccine using a virulence-attenuated *S. typhi* (TAB) has been developed (Kumar *et al.* 1974). Since *Salmonella* strains readily colonize the gut mucosal surface (Carter and Collins, 1974), interact with Peyer's patches (PP) (McGhee and Metecky, 1989), and persist in GALT prior to reaching systemic tissues, *Salmonella* strains are being extensively investigated as oral vaccine strains.

Transformed cells may be selected using various techniques including screening by differential hybridization, identification of fused reporter gene products, resistance markers, anti-antigen antibodies, and the like. After identification of an appropriate clone, cells may be selected and cultivated under conditions appropriate to the circumstances, as for example, conditions favoring expression.

Another aspect of the invention is a method of preparing heterologous polypeptides. The method generally involves preparing one or more of the recombinant vectors herein disclosed, transforming a host cell with the recombinant vector, then selecting a vector containing host cell clone and finally isolating from the clone the desired polypeptide which will be a heterologous protein. Examples of useful proteins that might be used in preparing the recombinant vector include APase, cholera toxin B subunit, influenza hemagglutinin and neuraminidase epitopes, fragments of these proteins, or other desired proteins such as *E. coli* heat labile or

heat stable toxins, tetanus and pertussis toxins, botulinum and *Pseudomonas* exotoxin A, *Yersinia pestis* pestin and Vw antigen, listerolysis O, *Mycobacterium tuberculosis* membrane proteins or membrane proteins from any pathogenic or related bacterium, also proteins which mediate attachment to mucosal surfaces such as but not limited to *E. coli* type 1 fimbriae, or mediate bacterial uptake into eucaryotic cells such as but not limited to the *Shigella flexneri ipaBCD* proteins, protective antigens of pathogenic bacteria which are established or will become known.

Unlike gene fusions which are incorporated into the flagellin gene, or expressed fused to *lamB* described in the prior art, much larger pieces of heterologous gene sequences can be displayed on the *Salmonella* surface with the present invention. Flagellin gene fusions are restricted to small epitopes which will not interfere with flagellar export and assembly at the cell surface.

In contrast, relatively large segments of heterologous fusion proteins representing a large number of epitopes can be exported and displayed by this invention. The *bla* gene described in the prior art may have a similar capacity to export large heterologous fusion proteins, but the size limit has not been determined.

A clear advantage of the present invention is that in *Salmonella*, the expression of fusion proteins will be under normal *Salmonella* regulation. This will abrogate problems with overproduction lethality which is often observed in the inferior systems described in the prior art. This is of extreme importance with the expression of membrane proteins, and is a feature of the present invention which is clearly lacking in systems such as *bla* or *lamB* fusion systems.

Depending on the particular recombinant vector selected for transforming a host cell, recombinant heterologous polypeptides will be expressed in different areas of the cell. For those heterologous polypeptides expressed on the periplasmic face of the cytoplasmic membrane, or within the periplasmic space itself, isolation of the heterologous polypeptide may be achieved by cell lysis and other well-known procedures utilized in the isolation of a desired fusion protein. Heterologous fusion proteins exported to or through the outer membrane of the host cell may be isolated from the membranes themselves. Typical procedures include separation of inner and outer cell membranes by procedures such as sonication, disruption by high pressure (French press), disruption by chaotropic agents or detergents, followed by enzymatic treatment to remove RNA and/or DNA. After cellular disruption, membrane fragments can be isolated by ultracentrifugation to pellet the membrane fraction or alternatively membranes can be isolated on sucrose density gradients. Inner and outer membranes can be isolated by selective solubilization of the inner membrane by detergents or via sucrose density gradient centrifugation. Ready isolation of the fusion peptide from membranous material, is also easily achieved by methods well-known to those of skill in the art.

In certain embodiments, antigenic proteins are expressed on or close to the outer surface of the host cell. This allows a wide range of uses, ranging from vaccine preparation to assays for screening of epitopes particularly effective for stimulating an immune response. Selected epitopes of eukaryotic, viral, or prokaryotic pathogens expressed on the surface of a host cell may be used for vaccine development. It is contemplated that tumor specific genes may also be expressed and utilized to stimulate an immune response. Whole cells expressing immunogenic epitopes might be used

for agglutination-based screening tests. Surface expressed polypeptides of microorganisms would be readily identified by screening recombinant libraries for specific surface expressed polypeptides.

In one particular aspect of the invention, cholera toxin B subunit is expressed on the surface of a *Salmonella* transformed host cell using the new pZIP-OUT vector. When expressed from *Salmonella* strain TA2362 harboring pRSP-18 (FIG. 2), cholera toxin B subunits reacts to antisera, and exhibits agglutination, indicating exposure of epitopic regions on the external membrane surface of the whole cell.

Yet another aspect of the invention involves the preparation of vaccines. Antigens or epitopes of the desired antigen are selected and a gene encoding that antigen or epitopes of that antigen is inserted into one or more of the recombinant vectors disclosed. Appropriate host cells are transformed and after screening for transformants one is selected expressing the antigen or part of the antigen for which a vaccine is desired.

Vaccines may be prepared by any of a number of ways. For example, surface expressed antigens on host cells of the present invention may be safely ingested and are suitable for oral administration. Orally administered attenuated *Salmonella* cells, as discussed, are known to engender a good immunogenic response due to stimulation of a reaction through interaction with gut mucosa.

Likewise, cell fragments or subcellular fractions containing the membrane-bound antigen prepared in accordance with the present invention, may be safely ingested and are also suitable for oral administration. This would be particularly desirable in the case of

immunocompromised or immunosuppressed subjects wherein administration of the attenuated *Salmonella* cells themselves are unsuitable for oral administration.

Alternatively, cells, cell fragments, or subcellular fractions containing the membrane-localized antigen may be injected into a mammal to generate an immune response.

In both immunodiagnostics and vaccine preparation, it is often possible and indeed more practical to prepare antigens from segments of a known immunogenic protein or polypeptide. Certain epitopic regions may be used to produce responses similar to those produced by the entire antigenic polypeptide. Often responses to epitopic regions are not so strong as responses to the entire polypeptide. The epitopic regions if expressed on the outer membrane surface of a bacterial host cell are expected to generate immunogenic responses that are enhanced over responses of the free epitopic region.

Antigenic peptides expressed on bacterial host cell surfaces may be significant in developing vaccines to such important antigens as cholera toxin B subunit, influenza HA and N (neuraminidase), various HIV antigens, as well as but not limited to antigens which could offer protection to established infectious agents such as *Borrelia* sp. (the agent of Lyme disease), *Mycobacterium tuberculosis*, *M. leprae*, other mycobacteria such as those associated with immunosuppressed states (*e.g.*, *M. avium* and *M. intracellulare*), *Mycoplasma pneumoniae* and other pathogenic mycoplasmas, *Neisseria gonorrhoeae* and *N. meningitidis*, *Pseudomonas aeruginosa* and other pathogenic or opportunistic pseudomonads, *Legionella pneumophila*, pathogenic obligate intracellular bacteria (*e.g.*, *Rickettsia* sp., *Chlamydia* sp., *Ehrlichia* sp. *Coxiella* sp., also pathogenic *Yersinia* sp. (*e.g.*, *Y. pestis*, *Y. pseudotuberculosis*, *Y. enterocolitica* and related

species), *Pasteurella* sp., *Francisella* sp., *Bordetella* sp., *Nocardia* sp., *Campylobacter jejuni* and related species, *Helicobacter pylori*, *Treponema pallidum* and other treponemas, *Leptospira* sp., *Listeria monocytogenes*, *Actinomyces* and related species, *Haemophilus influenzae* and related species, *Brucella abortus*, pathogenic *Shigella* species, pathogenic Gram-positive species (*e.g.*, *Staphylococcus aureus*, *Streptococcus pyogenes*, as well as Group B, Group G and related forms, *Acinetobacter* sp., *Corynebacterium diphtheria*, anaerobic bacteria such as *Bacteroides fragilis*, pathogenic *Clostridium* sp., including *C. difficile*, *C. perfringens* and others, pathogenic anaerobic cocci (*e.g.*, *Peptostreptococcus* and *Veillonella* sp.) and pathogenic *E. coli* species. It is anticipated that these methods could be used for both but not limited to human and animal pathogens.

It is also contemplated that these methods could be used to develop vaccines to various pathogenic viruses. These include but are not limited to Alphaviruses (Togaviride), Flaviviruses, Picornaviruses, Bunyaviruses, Arenaviruses, Orthomyxoviruses, Paramyxoviruses, Coronaviruses, Rhabdoviruses, retroviruses, rotaviruses, reoviruses, orbiviruses, parvoviruses, Norwalk agent, papovaviruses, adenoviruses, herpes viruses, poxviruses, and hepatitis viruses.

These methods could also be used to produce novel vaccines against fungal and parasitic pathogens such as: agents which mediate deep mycoses, subcutaneous, cutaneous, and superficial mycoses. Also, these methods could produce vaccines to protozoa such as *Dientamoeba*, *Trichomonas vaginalis* and others, hemoflagellates, malarial species, *Toxoplasma gondii*, *Pneumocystis carinii*, helminths, schistosomes, trematodes, cestodes and nematodes including filarial nematodes.

It is also anticipated that the methods of the present invention will useful for vaccine development to emerging infectious agents such as Ebola virus, Hanta virus and others (Baron, 1991).

In other embodiments, the invention concerns primers capable of priming amplification of selected portions of disclosed DNA segments. As such, it is contemplated that oligonucleotide fragments corresponding to the sequences of SEQ ID NO:1 and SEQ ID NO:2 for stretches of between about 10 nucleotides to about 20 or to about 30 nucleotides will find particular utility, with even longer sequences, *e.g.*, 40, 50, 100, even up to full length, being more preferred for certain embodiments. The ability of such nucleic acid probes to specifically hybridize to *Salmonella* sequences will enable them to be of use in a variety of embodiments. For example, the probes can be used in a variety of assays for detecting the presence of complementary sequences in a given sample. However, other uses are envisioned, including the use of the sequence information for the preparation of mutant species primers, or primers for use in preparing other genetic constructions.

Nucleic acid molecules having stretches of 10, 20, 30, 50, or even of 100 nucleotides or so, complementary to SEQ ID NO:1 and SEQ ID NO:2 will have utility as hybridization probes. These probes will be useful in a variety of hybridization embodiments, such as Southern and Northern blotting in connection with analyzing surface- and periplasmic-directed fusion proteins and/or polypeptides including those having either structural or regulatory function in diverse organisms and in various strains and/or species. The total size of fragment, as well as the size of the complementary stretch(es), will ultimately depend on the intended use or application of the particular nucleic acid segment. Smaller fragments

will generally find use in hybridization embodiments, wherein the length of the complementary region may be varied, such as between about 14 and about 100 nucleotides, or even up to full length according to the complementary sequences one wishes to detect.

The use of a hybridization probe of about 14 nucleotides in length allows the formation of a duplex molecule that is both stable and selective. Molecules having complementary sequences over stretches greater than 14 bases in length are generally preferred, though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having gene-complementary stretches from about 15 to 20 or 30 nucleotides, or even longer where desired. Such fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, by application of nucleic acid reproduction technology, such as the PCR™ technology of U.S. Patent 4,683,202 (herein incorporated by reference) or by introducing selected sequences into recombinant vectors for recombinant production.

Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template or where one seeks to isolate surface-directed polypeptide or protein-encoding sequences from related species, functional equivalents, or the like, less stringent hybridization conditions will typically be needed in order to allow formation of the heteroduplex. In these circumstances, one may desire to employ conditions such as about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20°C to about 55°C. Cross-hybridizing species can thereby be readily identified as positively hybridizing

signals with respect to control hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

In certain embodiments, it will be advantageous to employ nucleic acid sequences of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal. In preferred embodiments, one may desire to employ a fluorescent label or an enzyme tag, such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmental undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known which can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples.

In general, it is envisioned that the hybridization probes described herein will be useful both as reagents in solution hybridization as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes under desired conditions. The selected conditions will depend on the particular circumstances based on the particular criteria

required (depending, for example, on the G+C content, type of target nucleic acid, source of nucleic acid, size of hybridization probe, *etc.*). Following washing of the hybridized surface so as to remove nonspecifically bound probe molecules, specific hybridization is detected, and quantitated if desired, by means of the label.

The nucleic acid segments of the present invention, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol.

It will be understood that this invention is not limited to the particular nucleic acid and amino acid sequences of SEQ ID NO:1 and SEQ ID NO:2. Therefore, DNA segments prepared in accordance with the present invention may also encode biologically functional equivalent proteins or peptides which have variant amino acids sequences. Such sequences may arise as a consequence of codon redundancy and functional equivalency which are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged.

Primers may be utilized for several purposes. For example, primers may be used to amplify selected portions of the disclosed DNA segments. Certain combinations of the amplified segments may prove more effective than others in efficiently targeting to inner or outer periplasmic membranes. Additionally, primers prepared from the disclosed DNA may be used to amplify regions of DNA from other related organisms in order to identify similar targeting sequences.

Once amplified products are obtained, they may be used as probes to detect and isolate selected DNA fragments using hybridization procedures. Like primers, probes may be DNA or RNA and are generally of similar size usually including at least a 10 nucleotide segment or more, often 15-25 base pairs. Probes may be labeled, for example, by radiolabeling, to assist in identification of nucleic acid sequences.

As part of the invention, kits useful for the expression of fusion proteins are also envisioned comprising separate containers, each having suitably aliquoted reagents for performing the foregoing methods. For example, the containers may include one or more *Salmonella* species or *E. coli* transformed to express an immunogenic protein either periplasmically or associated with the inner periplasmic membrane or associated with the outer membrane of the selected microorganism, either as whole cell preparations or as membrane fragments that include the desired immunogen. Particularly valuable embodiments will be those that provide immunogenic response to epidemic diseases such as cholera, influenza, human immunodeficiency virus and so forth. A particularly preferred embodiment is attenuated *Salmonella* harboring cholera toxin B subunit. Also included is a pharmaceutical vehicle for mixing with the cell or membrane fragments, pr ferably a liquid that is

suitable for oral administration. Such liquids have been previously discussed herein.

Suitable containers might be vials made of plastic or glass, various tubes such as test tubes, metal cylinders, ceramic cups or the like. Containers may be prepared with a wide range of suitable aliquots, depending on applications and on the scale of the preparation. Generally this will be an amount that is conveniently handled so as to minimize handling and subsequent volumetric manipulations. Most practitioners will prefer to select suitable endonucleases from common supplies usually on hand; however, such restriction endonucleases may also be optionally included in a kit preparation.

Vectors, whole cells, membrane preparations, or membrane fragments containing the immunogenic fusion proteins supplied in kit form are preferably supplied in lyophilized form, although such cells, membrane preparations or membrane fragments may also be taken up in a suitable pharmaceutically-acceptable vehicle such as aqueous ethanol solutions, glycols, syrups, elixirs, or the like and supplied as solutions, suspensions, or colloids.

The kits of the invention may comprise distinct container means for each component, or multiple container means. The kits of the present invention may also include a means for containing the individual containers in close confinement for commercial sale, such as, *e.g.*, injection or blow-molded plastic containers into which the desired vials or container means are retained.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Immunoblot analysis of outer membrane preparations of *Salmonella* harboring pAH13. Immunoblot probed with an anti-gp120 polyclonal antibody showing a *Salmonella*::PhoA::gp120/gp41 fusion protein. Lane A contains *E. coli* DH5α™ control; lane B, *E. coli* DH5α™ harboring pAH13; lane C, *S. typhimurium* TA2362; lane D, TA2362 harboring pAH13; lane E CEM cells infected with HIV213; and lane F, CEM cells. The location of the predicted 84-kDa fusion protein is indicated in lanes B and D (*). The HIV gp120 polypeptide is shown in HIV-infected CEM cells (#).

FIG. 2. Schematic for the construction of the *ctxB* fusion vector pRSP-18. pRSP-18 was constructed by first eliminating the *Ssp*I site between *Eco*RI and *Pst*I of pRIT10810. In the second step a *Hin*dIII/*Pvu*II fragment containing *Salmonella* expression and export signals (Sal') of the *phoA* fusion from pZIP-OUT was purified and ligated to a *Hin*dIII/*Ssp*I digest of pRIT10810. pSP18 containing '*ctxB* fused in frame with '*phoA*' resulted from blunt ended ligation of the *Pvu*II and *Ssp*I sites. Since pSP18 conferred only low level resistance to tetracycline a cassette containing the kanamycin resistance gene was introduced at the *Bam*HI site resulting in pRSP-18. The tribrid *ctxB* fusion is under control of the *Salmonella* expression and export signals of pZIP-OUT.

FIG. 3. Schematic for the construction of the *ctxB* fusion vector pIMB13. pIMB13 was constructed by first eliminating the *Ssp*I site in the vector portion of pZIP-IN by deleting an *Eco*RV/*Ssp*I fragment. In the second step an *Ssp*I fragment containing '*ctxB* from pRIT10810 was introduced at the *Ssp*I site of pZIP-IN. Blunt-ended ligation resulted in pIMB13, which contains a fusion of

'ctxB in frame with the *'phoA'* gene and *Salmonella* expression and export signals (*SalI'*) of pZIP-IN.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to nucleic acid segments encoding particular proteins, peptides, or polypeptides capable of forming fusion proteins that export to particular areas of a host cell. These nucleic acid segments are useful in constructing vectors that allow expression of heterologous proteins from appropriately transformed host cells. Polypeptides may be localized within the inner membrane/periplasmic space or exported to the cell membrane surface. Antigens or epitopic regions of antigens localized on host cell membranes have particular potential for vaccine development and antibody production.

Construction of an Export-Directing Vector

A novel plasmid vector, pZIP-OUT, has been developed that through the production of fusion polypeptides directs the products of heterologous genes to the surface of the *Salmonella* outer membrane (OM). An important element of this plasmid is a sequence that provides the proper motif for the export of polypeptides to the surface of the OM. pZIP-OUT2 is a derivative of this plasmid which has a single *PvuII* site within the *phoA* gene.

To document that the plasmid-encoded *Salmonella* export sequences directs a fusion protein to the OM, subcellular fractions of *Salmonella* harboring pZIP-OUT were prepared to yield total envelope (TE), OM and inner membrane (IM) preparations (Curtiss *et al.*, 1986). These were analyzed by immunoblotting by probing with a monoclonal antibody (MoAb) to APase (CalTag Inc., South

San Francisco, CA). An APase fusion protein was observed in pZIP-OUT-containing cells that was present at high levels in the OM and TE, while only trace amounts were present in the IM.

In related studies, whole cell extractions enriched for surface proteins were prepared and surface-exposed polypeptides were extracted from *Salmonella* harboring pZIP-OUT by mild urea extraction (Foulaki and Gruber, 1989). Ten ml of overnight stationary phase bacterial cultures grown in LB broth were cooled on ice for 10 minutes and pelleted at 7,000 x *g*. The bacterial pellet was washed 3X in phosphate buffered saline (PBS). The washed pellet was resuspended in 0.1 ml of 6 M urea containing 10 mM Tris-HCl, pH 7.5 and 5mM EDTA. The suspension was incubated on ice for 20 min. Bacteria were pelleted at 12,000 x *g* in a microcentrifuge. Supernatants were removed and frozen at -20°C for analysis of surface proteins.

Immunoblot analysis of these surface extracts probed with a MoAb to APase showed pZIP-OUT mediated surface expression of an APase fusion protein, indicating that the APase fusion protein is directed to the OM surface.

DNA Sequence Determination

DNA sequence across the *phoA* fusion joint upstream into *Salmonella* sequence was determined for pZIP-OUT. A promoter (underlined) and Shine-Dalgarno sequence (boxed) were identified. The derived amino acid sequence contains an export specifying lipoprotein cleavage sequence (Leu-Ile-Gly-Cys), which is similar with other prokaryotic lipoprotein signal sequences (Wu and Tokunaga, 1985). A DNA sequence having homology to the described *Salmonella* sequence has recently been identified (Altmeyer, et al., 1993) and designated *invH*.

The Utility of pZIP-OUT in Directing Membrane Expression of Target Gene Sequences

The pZIP-OUT export-directing sequence is also useful in facilitating membrane expression of a variety of target genes. In these studies, restriction sites within the *phoA* gene were utilized which allow maintenance of the correct reading frame to produce *Salmonella*:PHOA:X tribrid fusion proteins.

Using this approach, the *V. cholera ctxB* gene encoding the cholera toxin B subunit (CTB), was introduced into the *phoA* gene of pZIP-OUT (pRSP-18). A 32 kDa CTB fusion protein was observed in OM but not IM fractions of TA2362 harboring pRSP-18. Surface extracts from pRSP-18 harboring cells showed a 32 kDa-reactive band. Antisera to CTB agglutinated *Salmonella* harboring pRSP-18, but not this strain alone, indicated that epitopic region(s) of CTB expressed by pRSP-18 was exposed on the surface of the cell.

Production of CTB-antibodies in Strains Harboring pRSP-18 Constructs

Cultures of attenuated *Salmonella* bacteria are prepared for oral immunization using about 2×10^8 to 2×10^9 CFU in phosphate buffered saline (PBS) containing 3% $NaHCO_3$. Doses in this range have given optimal responses in the present studies, but may be adjusted based on the particular subject and delivery vehicles used. Oral feeding of CTB (10-500 μg) in PBS containing 3% $NaHCO_3$ is used as a control. Initial boosting occurs at 18-21 days postchallenge, with subsequent boosting as necessary. Mucosal and serum antibodies to CTB are determined after primary challenge and all subsequent immunizations. CTB-specific Ig is quantitated using established ELISA protocols (Lycke *et al.*, 1988) with CTB

or CT bound to GM1 ganglioside-coated plates. Antibody levels to the *Salmonella* carrier strain are also determined. Titers to *Salmonella* are determined by a whole cell ELISA using *Salmonella* glutaraldehyde-fixed to microtiter wells. Mucosal Ig responses are determined from extracts of intestinal scrapings (Van der Heijden *et al.*, 1991) by ELISA. Horseradish peroxidase-(HRP) or AP-conjugated monoclonal antibodies are used for detection. Commercially available conjugated Ig-specific monoclonal antibodies to IgG, IgM and IgA facilitate determination of the isotype of the CTB-specific antibodies.

Development of pZIP-OUT

A heterologous gene expression system has been developed which utilizes a virulence-attenuated *Salmonella* as a carrier for a plasmid expression system (pZIP-OUT) which can direct the products of large segments of heterologous genes to the outer membrane. Recombinant DNA techniques are utilized to fuse the reading frame of the gene to be expressed with *Salmonella* export specifying sequences. Several cloning sites are possible which allow maintenance of the proper reading frame and produce tribrid fusion polypeptides which contain *Salmonella* export specifying sequences, the heterologous gene sequences and *phoA* gene sequences. Recombinants which export the tribrid fusion protein are selected through the loss of *phoA* activity and appearance of the predicted fusion polypeptide on the surface of the outer membrane. A tribrid fusion was constructed that encodes virtually the entire cholera toxin B subunit (*ctxB*) gene, and its subcellular localization in *Salmonella* was determined. This fusion polypeptide is expressed on the *Salmonella* surface as evidenced by: 1) agglutination of tribrid fusion expressing strains by anti-CTB antiserum, 2) localization of the fusion

polypeptide in the outer membrane, and 3) the presence of the fusion polypeptide in cell surface preparations.

DNA encoding the exported polypeptide of the present invention was isolated from *S. typhimurium*, strain TA2362, a virulence-attenuated strain. Virulence attenuated refers to species that have lost one or more virulence factors. In some *Salmonella* strains the loss of large plasmids is associated with loss of virulence, while in other strains chromosomal determinants of virulence appear to be involved. Isolation of an attenuated strain of *Salmonella* was useful in developing the present invention because such strains may be used to deliver heterologous antigens to the gut of an animal. *Salmonella* given orally tends to establish an infection in the intestinal mucosa, leading to an immune response.

The approach to screening for protein export signals was to use APase fusions based on the Tn*phoA* transposon system reviewed by Manoil *et al.* (1990). Tn*phoA* is a transposon derivative of Tn5 which lacks promoter, translation initiation site, signal sequence DNA and the first five amino acids of its protein. When the transposon, Tn*phoA*, inserts into a foreign gene in the correct orientation and reading frame, gene fusions are generated, coding for hybrid proteins which are subsequently expressed having APase activity. Detection of such activity is generally accomplished with a phosphatase-specific chromogenic substrate, such as 5-bromo-4-chloro-3-indolyl phosphate (X-P), allowing visualization of colored colonies for successful gene fusions that lead to export of heterologous gene products.

Vaccine Preparation and Use

Vaccines are the most cost effective medical intervention known to prevent disease. However, effective vaccines are available for relatively few diseases. Successful immunization against infectious organisms often requires a multicomponent host immune response against a variety of antigenic determinants. Orally administered vaccines, especially live attenuated vaccines, induce specific cell-mediated effector responses and elicit secretory IgA (sIgA) responses. sIgA is important because of its effectiveness at mucosal surfaces. sIgA production and cell effector responses are mediated through the delivery of antigens to gut-associated lymphoid tissue (GALT). Stimulation of GALT can lead to effective cell and humoral defense at all mucosal surfaces and provide systemic protection (Majarian *et al.*, 1989; Metecky, 1987).

To deliver antigens to GALT, investigators have developed avirulent and virulence-attenuated *Salmonella* stains. Aromatic dependent *aroA* (Sadoff *et al.*, 1988), *phoN* (Poirer *et al.*, 1988), *galE* (Clements, 1989), and *cya/crp* (Brown *et al.*, 1987) *Salmonella* mutants have been reported to interact with GALT in the lamina propria and stimulate an immune response. While it is clearly desirable to use avirulent *Salmonella* strains as carriers for plasmids which express protective antigens of other pathogens on their surface, there has been little success in developing protective vaccines based on this system.

The present invention includes vaccine preparation and use. Antigens, or epitopes of antigens, are readily expressed in localized regions of a host cell using the methods disclosed. Expression vectors incorporating the DNA segment encoding exportation polypeptides direct products to a host cell outer membrane surface. Epitopic

regions of antigens, well exposed at a membrane surface, as demonstrated with cholera toxin subunit B, elicit immunogenic responses, providing a route to vaccines or antibody production.

General concepts related to methods of vaccine preparation and use are discussed as applicable to preparations and formulations with antigens, epitopes or sub-fragments of such antigens obtained from various sources. Although virtually any antigen is contemplated, specific examples of antigens may include cholera toxin B subunit, influenza hemagglutinin, influenza neuraminidase, rickettsial outer membrane (OM) p190 protein, bacterial endotoxins, exotoxins, and the like.

Preparation of vaccines which contain peptide sequences as active ingredients is generally well understood in the art, as exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770, all incorporated herein by reference. Typically, such vaccines are prepared as injectables either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccines.

Vaccines are preferably administered orally, although parenterally, by injection, or either

subcutaneously or intramuscularly is also contemplated. Additional formulations which are suitable for other modes of administration include suppositories and oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10-95% of active ingredient, preferably 25-70%.

Proteins may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include acid addition salts (formed with the free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, *e.g.*, the capacity of the individual's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered depend on

the judgment of the practitioner. However, suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination. Suitable regimes for initial administration and booster shots are also variable but are typified by an initial administration followed by subsequent inoculations or other administrations. One will select particular avirulent strains depending upon the individual requirements of the subject receiving the vaccine.

Likewise, the manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable, particularly oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion. Alternatively, administration may be parenterally, by injection or the like. The dosage of the vaccine will depend on the route of administration and will vary according to the size of the host.

Various methods of achieving adjuvant effect for the vaccine include use of agents such as aluminum hydroxide or phosphate (alum), commonly used as 0.05 to 0.1 percent solution in phosphate buffered saline, admixture with synthetic polymers of sugars (Carbopol™) used as 0.25 percent solution, aggregation of the protein in the vaccine by heat treatment with temperatures ranging between 70° to 101°C for 30 second to 2 minute periods respectively. Aggregation by reactivating with pepsin treated (Fab) antibodies to albumin, mixture with bacterial cells such as *C. parvum* or endotoxins or lipopolysaccharide components of gram-negative bacteria, emulsion in physiologically acceptable oil vehicles such as mannide mono-oleate (Aracel A™) or emulsion with a 20 percent solution of a perfluorocarbon (Fluosol-DA™) used as a block substitute may also be employed.

In many instances, it will be desirable to have multiple administrations of the vaccine, usually not exceeding six vaccinations, more usually not exceeding four vaccinations and preferably one or more, usually at least about three vaccinations. The vaccinations will normally be administered from two to twelve week intervals, more usually from three to five week intervals. Periodic boosters at intervals of 1-5 years, usually three years, will be desirable to maintain protective levels of the antibodies. The course of the immunization may be followed by assays for antibodies for the supernatant antigens. The assays may be performed by labeling with conventional labels, such as radionuclides, enzymes, fluorescers, and the like. These techniques are well known and may be found in a wide variety of patents, such as U.S. Patent Nos. 3,791,932; 4,174,384 and 3,949,064, as illustrative of these types of assays. Alternatively, the course of the immunization may be followed by intestinal lavage with quantitation of the attenuated cells containing the surface-directed antigen.

Host Cells and Construction of Expression Vectors

The invention also contemplates the use of disclosed nucleic acid segments in the construction of expression vectors or plasmids and use in host cells. The following is a general discussion relating to such use and the particular considerations in practicing this aspect of the invention.

In general, of course, prokaryotes, and in particular *Proteobacteria*, are preferred for the initial cloning of DNA sequences and constructing the vectors useful in the invention. Most preferred are members of the family *Enterobacteraceae*. For example, in addition to the particular genera mentioned in the more specific disclosure below, one may mention by way of example,

genera such as *Escherichia, Salmonella, and Shigella*. More preferably strains such as *E. coli* K12 strain 294 (ATCC No. 31446), *E. coli* B, and *E. coli* X 1776 (ATCC No. 31537) are employed.

Prokaryotes are also preferred for expression, and more particularly, *Proteobacteria*, including strains of such species as *E. coli* W3110 (F-, lambda-, prototrophic, ATCC No. 273325), or other *Enterobacteriaceae* such as *Salmonella typhimurium* or *Serratia marcescens*, and various *Pseudomonas* species. Additional Gram-negative bacteria are also envisioned to be useful in the expression of the vectors of the present invention. Likewise, bacilli such as *Bacillus subtilis* are also contemplated. These examples are, of course, intended to be illustrative rather than limiting.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (see, *e.g.*, Bolivar *et al.*, 1977). The pBR322 plasmid contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters which can be used by the microorganism for expression.

Those promoters most commonly used in recombinant DNA construction include the β-lactamase (penicillinase) and lactose promoter systems (*e.g.*, β-galactosidase) (Chang *et al.*, 1978; Itakura *et al.*, 1977; Goeddel *et al.*, 1979) and a tryptophan (*trp*) promoter system

(Goeddel *et al.*, 1979; EPO Appl. Publ. No. 0036776). While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (Siebwenlist *et al.*, 1980). Certain genes from prokaryotes may be expressed efficiently in *E. coli* from their own promoter sequences, precluding the need for addition of another promoter by artificial means.

In addition to prokaryotes, eukaryotic microbes, such as yeast cultures may also be used. *Saccharomyces cerevisiae*, or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are available. For expression in *Saccharomyces*, the plasmid YRp7, for example, is commonly used (Stinchcomb *et al.*, 1979; Kingsman *et al.*, 1979; Tschemper *et al.*, 1980). This plasmid already contains the *trpL* gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, 1977). The presence of the *trpL* lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzman *et al.*, 1980) or other glycolytic enzymes (Hess *et al.*, 1968; Holland *et al.*, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated

with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination.

Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Any plasmid vector containing a yeast-compatible promoter, origin of replication and termination sequences is suitable.

In addition to prokaryotic organisms, cultures of eukaryotic cells derived from multicellular organisms may also be used as hosts in both the construction and expression of aspects of the present invention. In principle, any such cell culture is useful, whether from vertebrate or invertebrate culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (Tissue Culture, 1973). Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7 293 and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently

Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers *et al.*, 1978). Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the *HindIII* site toward the *BglI* site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (*e.g.*, Polyoma, Adeno, VSV, BPV) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

Also contemplated within the scope of the present invention is the use of the disclosed DNA as a hybridization probe. While particular examples are provided to illustrate such use, the following provides general background for hybridization applications taking advantage of the disclosed nucleic acid sequences of the invention.

Nucleic Acid Hybridization Embodiments

In certain aspects, the DNA sequence information provided by the invention allows for the preparation of relatively short DNA (or RNA) sequences having the ability to specifically hybridize to *S. typhimurium* gene sequences. In these aspects, nucleic acid probes of an appropriate length are prepared based on a consideration

of the sequence, *e.g.*, as shown SEQ ID NO:1 and SEQ ID NO:2 or derived from flanking regions of these genes. The ability of such nucleic acid probes to specifically hybridize to the *S. typhimurium* gene sequences lend them particular utility in a variety of embodiments. The probes can be used in a variety of diagnostic assays for detecting the presence of pathogenic organisms in a given sample. However, other uses are envisioned, including the use of the sequence information for the preparation of mutant species primers, or primers for use in preparing other genetic constructs.

To provide certain of the advantages in accordance with the invention, the preferred nucleic acid sequence employed for hybridizations or assays includes sequences that are complementary to at least a 10 to 40, or so, nucleotide stretch of the selected sequence, such as that shown in SEQ ID NO:1 or SEQ ID NO:2, respectively. A size of at least 10 nucleotides in length helps to ensure that the fragment will be of sufficient length to form a duplex molecule that is both stable and selective. Molecules having complementary sequences over stretches greater than 10 bases in length are generally preferred, though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. Thus, one will generally prefer to design nucleic acid molecules having gene-complementary stretches of 15 to 20 nucleotides, or even longer where desired. Such fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, by application of nucleic acid reproduction technology, such as the PCR™ technology of U.S. Patent 4,683,202, or by introducing selected sequences into recombinant vectors for recombinant production.

The present invention is envisioned as useful in the cloning of nucleic acids encoding certain exportation polypeptides. Identification of other exportation polypeptides in addition to the 50 kDa and 63 kDa proteins should be possible using methods analogous to those disclosed herein. One method would be to produce a cDNA library using mRNA obtained from mutant *S. typhimurium* strains. Although the production of cDNA libraries from bacteria is not commonly done because of the usual absence of poly-A tails on prokaryotic messages, a cDNA library may be constructed from *S. typhimurium* mRNA.

A method of preparing variants of the *S. typhimurium* exportation polypeptides is site-directed mutagenesis. This technique is useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, derived from the 50 kDa or 63 kDa protein sequence, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art as exemplified by

publications (Adelman *et al.*, 1983). As will be appreciated, the technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage (Messing *et al.*, 1981). These phage are readily commercially available and their use is generally well known to those skilled in the art.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector which includes within its sequence a DNA sequence which encodes an export polypeptide. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example by the method of Crea *et al.* (1978). This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected which include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected exportation polypeptide gene using site-directed mutagenesis is provided as a means of producing potentially useful exportation species and is not meant to be limiting as there are other ways in which sequence variants of the exportation polypeptide gene may be obtained. For example, recombinant vectors encoding the desired 30K gene may be treated with mutagenic agents to obtain sequence variants (see, *e.g.*, a method described

by Eichenlaub, [1979] for the mutagenesis of plasmid DNA using hydroxylamine).

Epitopic Core Sequences

Further embodiments of the present invention are thus contemplated to include protein compositions, free from total bacterial cells, characterized as being associated with outer membranes or fragments thereof and having immunogenic activity. Such proteins may either be isolated directly by cell fractionation and protein purification or may be produced by recombinant techniques following the molecular cloning of a DNA segment, as described herein.

The present invention is also directed to protein, peptide or polypeptide compositions, free from total bacterial cells, which comprise a purified protein, peptide or polypeptide which incorporates an epitope that is immunologically cross-reactive with a surface-directed fusion protein described herein.

As used herein, the term "incorporating an epitope(s) that is immunologically cross-reactive with a surface-directed fusion protein" is intended to refer to a peptide or protein antigen which includes a primary, secondary or tertiary structure similar to an epitope associated with the outer membrane or periplasmic space of *Salmonella* or *E. coli* of an export-directed fusion protein as described herein. The level of similarity will generally be to such a degree that monoclonal or polyclonal antibodies directed against the fusion protein will bind to, react with, or otherwise recognize, the cross-reactive peptide or protein antigen. Various immunoassay methods may be employed in conjunction with such antibodies, such as, for example, Western blotting,

Additionally or alternatively, an epitopic core sequence is one that will elicit antibodies that are cross-reactive with antibodies directed against the surface-expressed fusion proteins of the present invention. It will be understood that in the context of the present disclosure, the term "complementary" refers to amino acids or peptides that exhibit an attractive force towards each other. Thus, certain epitope core sequences of the present invention may be operationally defined in terms of their ability to compete with or perhaps displace the binding of the desired protein antigen with the corresponding protein-directed antisera.

In general, the size of the polypeptide antigen is not believed to be particularly crucial, so long as it is at least large enough to carry the identified core sequence or sequences. The smallest useful core sequence anticipated by the present disclosure would generally be on the order of about 15 amino acids in length. Thus, this size will generally correspond to the smallest peptide antigens prepared in accordance with the invention. However, the size of the antigen may be larger where desired, so long as it contains a basic epitopic core sequence.

Syntheses of epitopic sequences, or peptides which include an antigenic epitope within their sequence, are readily achieved using conventional synthetic techniques such as the solid phase method (*e.g.*, through the use of commercially available peptide synthesizer such as an Applied Biosystems Model 430A Peptide Synthesizer). Peptide antigens synthesized in this manner may then be aliquotted in predetermined amounts and stored in conventional manners, such as in aqueous solutions or, even more preferably, in a powder or lyophilized state pending use.

ELISA, RIA, and the like, all of which are known to those of skill in the art.

The identification of immunogenic epitopes, and/or their functional equivalents, suitable for use in vaccines is a relatively straightforward matter. For example, one may employ the methods of Hopp, as taught in U.S. Patent 4,554,101, incorporated herein by reference, which teaches the identification and preparation of epitopes from amino acid sequences on the basis of hydrophilicity. The methods described in several other papers, and software programs based thereon, can also be used to identify epitopic core sequences (*e.g.*, Jameson and Wolf, 1988; Wolf *et al.*, 1988; Hopp, U.S. Patent Number 4,554,101; and Kyte and Doolittle, 1982). The amino acid sequence of these "epitopic core sequences" may then be readily incorporated into peptides, either through the application of peptide synthesis or recombinant technology.

Preferred peptides for use in accordance with the present invention will generally be on the order of 15 to 50 amino acids in length, and more preferably about 15 to about 30 amino acids in length. It is proposed that shorter antigenic peptides which incorporate epitopes of the surface-expressed fusion proteins will provide advantages in certain circumstances, for example, in the preparation of vaccines or in immunologic detection assays. Exemplary advantages include the ease of preparation and purification, the relatively low cost and improved reproducibility of production, and advantageous biodistribution.

An epitopic core sequence, as used herein, is a relatively short stretch of amino acids that is "complementary" to, and therefore will bind, antigen binding sites on transferrin-binding protein antibodies.

In general, due to the relative stability of peptides, they may be readily stored in aqueous solutions for fairly long periods of time if desired, *e.g.*, up to six months or more, in virtually any aqueous solution without appreciable degradation or loss of antigenic activity. However, where extended aqueous storage is contemplated it will generally be desirable to include agents including buffers such as Tris or phosphate buffers to maintain a pH of 7.0 to 7.5. Moreover, it may be desirable to include agents which will inhibit microbial growth, such as sodium azide or Merthiolate. For extended storage in an aqueous state it will be desirable to store the solutions at 4°C, or more preferably, frozen. Of course, where the peptide(s) are stored in a lyophilized or powdered state, they may be stored virtually indefinitely, *e.g.*, in metered aliquots that may be rehydrated with a predetermined amount of water (preferably distilled) or buffer prior to use.

Isolation of *Salmonella* DNA Segments

Isolation of *Salmonella* DNA segments was accomplished by isolation of *pho*A/DNA fusions. Tn*phoA* is a derivative of Tn5 which encodes *E. coli* APase, minus the signal sequence and expression signals, inserted into the left *IS50L* element. Random transposition of Tn*phoA* results in an active insertion only when the *phoA* gene sequence is fused in-frame downstream of the promoter and export signals of a target gene A. Plasmids containing *phoA* gene fusions can then be used as exposition vectors (B). The *Ssp*I and the *Pvu*II restriction sites in *phoA* are blunt ended sites at which in-frame insertions (IF) of a gene of interest (GOI) can be inserted. The resulting tribrid gene fusions contain the expression and export signals of the target gene fused in-frame with the *phoA* and GOI sequences.

The following examples are intended to illustrate the practice of the present invention and are not intended to be limiting. Although the invention is demonstrated with nucleic acid segments isolated from a strain of *Salmonella*, similar functions may be obtained from nucleic acid segments from other *Salmonella* species and strains, other *Enterobacteraceae*, and even other bacteria and higher microorganisms. The nucleic acid sequences identified and the corresponding encoded polypeptides are useful in developing methods of producing a wide variety of heterologous proteins as well as expression vectors for localizing polypeptides in selected areas of a host cell.

It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those skilled in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

The following example illustrates construction of plasmid pZIP-IN, as disclosed in U.S. Patent 5,356,797, the entire text and figures of which are specifically incorporated herein by reference in their entirety. This plasmid is basically a chimeric gene including a *Salmonella* DNA segment fused with a segment of an APase gene lacking signal and expression sequences. When expressed in a suitable host cell, the fusion product is

localized to the inner membrane/periplasmic space of the host cell.

PREPARATION OF pZIP-IN FUSION VECTOR

pZIP-IN, is a derivative of pBR322 containing a *Bam*HI fragment encoding APase activity and kanamycin resistance inserted at the *Bam*HI site. The *Bam*HI fragment was cloned from a chromosomal DNA preparation of the Tn*phoA* insertion mutant TAG28 that was constructed by Tn*phoA* mutagenesis (see above) of *S. typhimurium* TA2361 (*phoN* mutant derived from LT2).

Chromosomal DNA was prepared from 50 ml of overnight growth of TAG28 in L-broth with vigorous shaking at 37°C. The bacterial culture was precipitated and washed once in phosphate buffered saline (pH 7.0). The washed bacterial pellet was resuspended in 10 ml of ice cold ET buffer (10 mM EDTA, 10 mM Tris-HCl, pH 8.0). Lysozyme was added to a concentration of 0.1 mg/ml and incubated for 15 minutes at 37°C. 1.2 ml of sarkosyl-pronase solution (10% sarkosyl, 5 mg/ml Pronase in ET buffer) was added and the solution was incubated for 1 hr at 37°C. The solution was then extracted 3 times with TE (10 mM Tris HCl, 1 mM EDTA, pH 8.0) saturated phenol followed by 3 extractions with chloroform:isoamyl alcohol (24:1). The aqueous phase was transferred to a 50 ml beaker on ice and one-half volume of 7.5 M ammonium acetate was added. Three volumes of ice cold absolute ethanol was gently layered on top of the solution. The chromosomal DNA was precipitated onto a glass rod by gently stirring the solution to mix the interface. The precipitated DNA was rinsed once in 70% ice cold ethanol and dissolved overnight in 2 ml of TE buffer at 4°C. The concentration of DNA was quantitated by measuring the O.D. at 260 nm.

2 μg of TAG28 chromosomal DNA was digested with *Bam*HI at 37°C for 2 hrs. The solution was extracted once with TE saturated phenol, followed by 2 extractions with chloroform:isoamyl alcohol (24:1). The aqueous phase was removed and the DNA precipitated by the addition of 1/10 volume 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol followed by centrifugation in a microcentrifuge. 0.2 μg of pBR322 DNA was digested with *Bam*HI and prepared for ligation as above. Ligation of the vector DNA (pBR322) and TAG28 chromosomal DNA was performed by overnight incubation at 4°C in 20 μl of 1X commercial (Promega) ligase buffer and 2 U of T4 DNA ligase.

pZIP-IN was isolated from the ligation reaction by transformation of DH5α™ (GIBCO-BRL, Bethesda, MD) competent cells. 5 μl of the ligation mixture was added to 50 μl of DH5α competent cells and incubated on ice for 30 minutes. Cells were heat shocked for 30 seconds by submerging in a 37°C water bath. Cells were cooled on ice for 2 minutes and 0.950 ml of L-Broth was added to the tube. Cells were incubated for 1 hr at 37°C. Transformants with APase activity and kanamycin resistance were selected by plating 0.1 ml of the bacterial culture on the L-agar plates containing 50 μg/ml kanamycin and 40 μg/ml X-P, followed by overnight incubation at 37°C. The following day, kanamycin resistant colonies were visible and all were blue, indicating that the transformants had APase activity. This was confirmed by APase assays, Western blotting with monoclonal antibodies to APase, and DNA sequencing of the fusion joint.

EXAMPLE 2

The following example illustrates the construction of pZIP-OUT, as disclosed in PCT Published Application Serial Number, WO 93/10246, the entire specification and figures of which are specifically incorporated herein by reference in their entirety. The plasmid was constructed from a DNA segment of *Salmonella* and a PhoA DNA segment lacking signal and expression sequences. When expressed from a host cell, the fusion protein localized to the host cell outer membrane fraction.

PREPARATION OF pZIP-OUT EXPRESSION VECTOR

Genomic DNA was isolated from *Salmonella* strain TAP43. A 25 ml culture in LB broth was grown overnight at 37°C with shaking. The cells were harvested by centrifugation, and the pellet washed once in PBS. The washed pellet was resuspended in 10 ml of cold TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). One ml of a 1 mg/ml lysozyme solution was added, and the mixture was incubated in a 37°C water bath for fifteen minutes. After this incubation, 1.2 ml of 10% sarkosyl, 5 mg/ml pronase in TE buffer was added, and incubation continued at 37°C for 1-2 hours, until cell lysis occurred. The lysate was then extracted twice with an equal volume of phenol, once with phenol/chloroform, and once with chloroform. To the final extraction, a half-volume of 7.5 M ammonium acetate was added. The solution was mixed gently and placed on ice. Two volumes of ice-cold absolute ethanol were layered on top of the lysate, and the chromosomal DNA was collected at the interface by spooling on a glass rod. The spooled DNA was rinsed once in 70% ethanol, and then allowed to dissolve off of the glass rod into TE buffer overnight at 4°C. The buffer, containing the dissolved DNA, was then ethanol-precipitated. The purified chromosome was collected by

centrifugation and resuspended in a small volume of TE buffer. 1-5 μg of the purified DNA was restricted with *Hind*III, and then phenol/chloroform extracted and ethanol precipitated. The sample was collected by centrifugation, the pellet washed once with 70% ethanol, and dried under vacuum.

Vector pUC18 was also restricted with *Hind*III, extracted, and precipitated in the same manner. The *Hind*III fragments of the genomic DNA were then ligated into the *Hind*III site of pUC18 with T4 DNA ligase. After ligation, the DNA was transformed into competent DH5α cells and plated on L-agar supplemented with ampicillin and X-P, both at 40 μg/ml. Blue colonies, indicating the presence of an active APase fusion in the transformant, were selected and analyzed by restriction mapping. Transformant #43-17 contained a 4.5 kb *Hind*III insert in the pUC18 vector. 3.1 kbp of this insert consisted of *phoA* sequences, with the remaining 1.4 kbp being derived from *Salmonella* chromosomal sequences. This construct was designated pZIP-OUT.

The identity of pZIP-OUT as a *phoA* fusion was confirmed by restriction analysis, Southern hybridization analysis, and finally, by DNA sequencing. The *Salmonella-phoA* fusion contained within this *Hind*III fragment was designated as the pZIP-OUT cassette. This cassette was subsequently cloned into the *Hind*III sites of the vectors pBR322 and pAT153.

EXAMPLE 3

The following example illustrates how DNA may be fused to the gene segments of plasmid pZIP-IN, shown in this example with a portion of the cholera toxin subunit B gene.

CONSTRUCTION OF pIMB13 USING THE pZIP-IN VECTOR

pIMB13 (FIG. 3) is a derivative of pZIP-IN in which the final 294 base pairs of *ctxB* have been inserted in frame with the *phoA* gene sequence at the *Ssp*I site. The inserted fragment containing the *ctxB* gene sequence is from pRIT10810 which encodes the entire *ctxB* gene. First, the *Ssp*I site in the pBR322 portion of pZIP-IN was eliminated as follows. 2 μg of a plasmid preparation of pZIP-IN was digested with *Sca*I and *Eco*RV. Both enzymes cut at a single site within the pBR322 portion of the vector and generate compatible blunt ends. The digested DNA was precipitated and ligation was performed in 20 μl of 1X ligase buffer containing 1 U of T4 DNA ligase overnight at 4°C. DH5α™ frozen competent cells were transformed with 5 μl of the ligation reaction mixture.

Transformants were selected on L-agar plates containing 50 μg/ml kanamycin. Colonies were then replicated to L-agar plates containing 40 μg/ml ampicillin. Loss of ampicillin resistance encoded by pZIP-IN indicated that the segment from ScaI (3844) to *Eco*RV (185) which contained the *Ssp*I site (4168) had been eliminated. The resulting plasmid pΔS28-1 contained a single *Ssp*I site in the *phoA* sequence which generates an in-frame blunt end cut.

pIMB13 was constructed from pΔS28-1 as follows. The *ctxB* sequence encoded by pRIT10810 contains an *Ssp*I site which generates an in-frame blunt end cut near the 5′ end of the structural gene. pRIT1080 also contains an *Ssp*I site in the pBR322 portion of the vector. Digestion of pRIT101810 with *Ssp*I generates 2 fragments, one of which contains the 3′ final 294 base pairs of *ctxB*. 2 μg of pΔS28-1 and 2 μg of pRIT10810 were digested with *Ssp*I. Following phenol/chloroform extraction, the samples were combined and precipitated with 2 volumes of ethanol.

Ligation of the sample was performed in 20 μl of 1X ligase buffer containing 1 U T4 DNA ligase. DH5α frozen competent cells were transformed with 5 μl of the ligation mixture. Transformants were selected on L-agar plates containing 50 μg/ml kanamycin and 40 μg/ml X-P. Colonies harboring pΔS28-1 with inserts at the *phoA* *Ssp*I site appeared white since insertion interrupted the active *phoA* gene fusion. White kanamycin resistant colonies were picked for isolation and screened for expression of a *ctxB* fusion protein by Western blotting of total envelope fractions with affinity purified anti-*ctxB*. A DH5α strain harboring a derivative of pZIP-IN encoding a *ctxB* gene fusion was identified and the plasmid was designated pIMB13.

EXAMPLE 4

The following example illustrates a tripartite fusion prepared from plasmid pZIP-OUT. This plasmid may be used to express a fusion polypeptide from suitable host cells. The DNA inserted in this example is a segment from cholera toxin B subunit.

CONSTRUCTION OF A TRIPARTATE FUSION USING pZIP-OUT

The construction of the trihybrid fusion, pRSP-18, was accomplished as follows. pRIT10810, containing the cholera toxin B gene, was first restricted with *Eco*RI and *Pst*I. The ends generated by these restrictions were repaired with Klenow, and the vector was ligated back together. This created a 0.8 kbp deletion in pRIT10810, eliminating an undesirable *Ssp*I site in the vector. This deleted pRIT10810 was then restricted with *Hind*III and *Ssp*I. pZIP-OUT (in vector pUC18) was doubly restricted with *Hind*III and *Pvu*II. A 2.0 kbp fragment generated from this double restriction, consisting of 1.4 kbp of *Salmonella* sequence and 0.6 kbp of *phoA*, was isolated and

purified after agarose gel electrophoresis. This 2.0 kbp fragment was then unidirectionally ligated into the *Hin*dIII/*Ssp*I digested pRIT10810. This generated an in-frame fusion of the *Salmonella-phoA* sequences to the *ctxB* sequence (pSP-18). This clone was selected on the basis of weak tetracycline resistance (1 μg/ml in L-agar). To make further manipulations of the plasmid more efficient, a kanamycin gene block (Pharmacia) was cloned into the *Bam*HI site of pSP-18, resulting in the plasmid construction pRSP-18.

EXAMPLE 5

This example illustrates the procedure for extracting and separating bacterial membranes. After isolation of the membrane fragments, they were analyzed for localization of fusion peptides.

PREPARATION OF BACTERIAL MEMBRANES (TOTAL ENVELOPE) AND SEPARATION INTO INNER AND OUTER MEMBRANE FRACTIONS

100 ml of overnight bacterial cultures grown in L-Broth with vigorous shaking were pelleted and washed 1X in phosphate buffered saline (pH 7.0). Washed pellets were resuspended in 3 ml of membrane isolation buffer [10 mM $NaPO_4$, 0.5 mM $MgSO_4$ (pH 7.0)]. Samples were sonicated for 20 seconds 3 times with cooling on ice in between. Unbroken cells were removed by centrifugation at 7,000 rpm. Then total envelopes were isolated in a Beckman ultracentrifuge using an SW55 rotor at 38,000 rpm for 1 hr. The supernatants were removed and total envelope pellets were rinsed 1X in sterile deionized water. Pellets were resuspended in 40 μl of sterile deionized water. One-half (20 μl) was saved for Western analysis of the total envelope. A 5% solution of sarkosyl in sterile deionized water was added to the remaining 20 μl to a final concentration of 0.5%. The samples were

incubated for 30 minutes at room temperature and centrifuged in a microcentrifuge to pellet the non-soluble fraction representing the outer membrane. The supernatant was removed for Western analysis of the inner membrane fraction. The outer membrane pellet was rinsed once in sterile deionized water and saved for Western analysis. Immunoblot analysis of membrane preparations using mouse anti-APase was performed.

EXAMPLE 6

The following example describes the analysis of APase activity. For purposes of the present invention, APase assays were performed to test for enzyme activity in membrane fractions of host cells in which alkaline fusion proteins were expressed.

DETERMINATION OF APase ACTIVITY OF FUSION PROTEINS

APase activity encoded by pZIP-IN and pZIP-OUT was confirmed by spectrophotometric assay using the chromogenic APase substrate *p*-nitrophenol phosphate (*p*NPP) (Table 1). One ml of overnight bacterial cultures was pelleted for 15 seconds in a microcentrifuge. The pellet was washed once in 1 M Tris-HCl (pH 8.0) and resuspended in 1 ml of 1 M Tris-HCl (pH 8.0). The optical density at 600 nm of the bacterial suspension was recorded. 50 μl of chloroform and 50 μl of 0.1% SDS were added to permeabilize the cells. Samples were vortexed briefly. 0.1 ml of a 0.4% solution of *p*-NPP in 1 M Tris-HCl (pH 8.0) was added and samples were incubated at 37°C. After development of a yellow color, 10 μl of 2.5 M KPO_4 and 0.5 M EDTA was added and samples were placed on ice to stop the reaction. Cellular debris was removed by centrifugation in a microcentrifuge. The optical density of the samples at 420 nm was recorded.

Alkaline phosphatase activity was determined as described (Beckwith and Manoil, 1984). Bacteria were grown under various conditions and washed once in Tris-buffered saline (pH 8.0). Optical density (O.D.) readings at A_{600} were determined for washed bacteria resuspended in alkaline phosphatase buffer (pH 9.5). 50 µl of SDS (0.1%) and chloroform each were added to permeabilize the cell suspension. After agitation, 100 µl of 0.4% *p*-NPP) was added. Cultures were incubated at 37°C until a substantial yellow color change occurred and 100 µl of 1 M KH_2PO_4 was added to stop the reaction. After incubation, samples were centrifuged to remove any cellular debris. Absorbance readings of samples were taken at A_{420}.

TABLE 1

ALKALINE PHOSPHATASE ACTIVITY OF THE ANTIGEN PLACEMENT VECTORS, pZIP-OUT AND pZIP-IN

Strain[a]	PhoA Activity[b] (Units/min)
E.coli CC118	11
E.coli CC118(pZIP-OUT)	1037
E.coli CC118(pZIP-IN)	652
E.coli CC118(pUC18)	13

[a] *E.coli* CC118 is Δ*phoA*. pZIP-OUT, pUC18 and pZIP-IN were introduced into CC118 by transformation. pUC18 is the parental plasmid of pZIP-OUT and pZIP-IN.

[b] Cultures were grown in L-broth overnight at 37°C with aeration and prepared for assay as reported (Beckwith and Manoil, 1985).
Activity was determined by:

$$Activity(units/\text{min}) = \frac{OD_{420}}{OD_{600} \; x \; time} \; x \; 1000$$

An immunoblot analysis of urea extracts using anti-APase as the primary antibody was performed. No reaction was shown with plasmid pZR322 or with plasmid pZIP-IN. A reaction was shown with plasmid pZIP-OUT, indicating extraction of the APase.

EXAMPLE 7

The following outlines the general procedure for extracting proteins from bacterial cells.

UREA EXTRACTION OF BACTERIAL CELLS

Ten ml of overnight stationary phase bacterial cultures grown in L broth with vigorous shaking were cooled on ice for 10 minutes and pelleted at 7,000 RPM in a Beckman J2-21 (JA-17 rotor). The bacterial pellet was washed 3 times in phosphate buffered saline (pH 7.0). The washed pellet was resuspended in 0.1 ml of 6 M urea containing 10 mM Tris-HCl (pH 7.5) and 5 mM EDTA. The suspension was incubated for 20 minutes on ice. Bacteria were pelleted in a microcentrifuge for 1 minute. Centrifugation of the supernatants was repeated to remove any traces of debris. Supernatants were frozen and 20 μl aliquots were used for SDS-PAGE and Western analysis.

EXAMPLE 8

The following example illustrates the expression of a cholera toxin subunit B (CtxB) polypeptide from an attenuated *Salmonella* strain with localization of the CtxB to the surface of the outer cell membrane.

PREPARATION OF SURFACE EXPRESSED CHOLERA TOXIN SUBUNIT B

The tribrid fusion in pRSP-18 contains a 1.4 kb *Salmonella* DNA sequence which encodes the expression export signals of the expressed gene. The *phoA* sequence of the fusion includes approximately 0.6 kb from the Tn*phoA* fusion joint (FJ) to the in-frame insertion (IF) of *ctxB*. The *ctxB* sequence includes the final 294 base pairs of *ctxB* beginning at the in-frame insertion site IF. Expression and export result in a 32 kDa tribrid

fusion protein including the final 98 amino acids of *ctxB* at the C-terminus which localizes to the outer membrane. The tribrid fusion in a pIMB13, contains a 1.3 kb *Salmonella* DNA sequence which encodes the expression and export signals of the expressed gene. The *phoA* sequence of the fusion includes approximately 0.2 kb from the Tn*phoA* fusion joint FJ to the inframe insertion IF of *ctxB*. The *ctxB* sequence includes the final 294 base pairs of *ctxB* beginning at the inframe insertion site IF. Expression and export result in a 32 kDa tribrid fusion protein including the final 98 amino acids of *ctxB* at the C-terminus which localizes to the inner membrane.

Whole *Salmonella* TA2362 cells harboring pRSP-18 were shown to express cholera B subunit on the outer surface membrane. Antisera to cholera toxin B subunit were prepared. Agglutination of TA2632 harboring pRSP-18 was obtained. No agglutination was observed with strain TA2362 alone, Table 2.

EXAMPLE 9

The following example illustrates the procedures contemplated as useful for creating an immune response in a mammal elicited with virulence attenuated *Salmonella* strains expressing antigens on the surface of the intact cell. In this example, CTB is used as an illustration.

IMMUNOGENIC RESPONSES FROM SURFACE-EXPRESSED CTB

Ten C57/Bl6 mice were orally challenged with a virulence-attenuated *S. typhimurium aroA phoN* strain, G361, harboring pRSP-18 and ten C57/Bl6 mice were challenged with the same straining harboring a plasmid which expresses CTB cytosolically (pRIT108010, ATCC-39051) produced CTB-specific antibodies after immunization (Table 2).

The results using I.P. challenge (5 x 10^5 cfu) and oral challenge (5 x 10^8 cfu) were evaluated. Boosting was 10 days post-challenge. Mucosal and serum anti-CTB levels were determined after 1° and 2° challenge by ELISA and by the ability to neutralize cholera toxin activity on adrenal cells (Majarian *et al.*, 1989). It was determined whether the membrane-expressed CTB tribrid polypeptide retained its potent mucosal adjuvant activity (Tagliabue, 1989) by comparing antibody titers to *Salmonella* and *Salmonella* expressing CTB. Since CTB mediates Ig class switching, IgA/IgG ratios were determined between the different challenge protocols by ELISA. Alternatively, the adjuvant activity of membrane expressed CTB were evaluated using a purified antigen (*i.e.*, ovalbumin) for concurrent challenge with *Salmonella* or *Salmonella* expressing CTB strains.

To account for the low expression of pRIT10810 in *Salmonella*, ~3X the bacterial challenge dose of this strain was utilized. While this may not represent an optimal comparison, significantly higher titers were observed after challenge with *Salmonella* harboring pRSP-18, the surface-encoded CTB strain. These data suggest that antigens present in different subcellular compartments are not equally "seen" by the immune system and that the placement of epitopes on the bacterial surface represent an optimal method for vaccine development.

TABLE 2

αCTB TITERS FROM MICE CHALLENGED ORALLY WITH *S. TYPHIMURIUM* EXPRESSING CTB IN VARIOUS SUBCELLULAR LOCATIONS

Plasmid[a]	Subcellular Location of Expressed CTB	ELISA Titer[b]
None		<4
pRIT10810	Cytosolic	17
pRSP-18	OM surface	210

[a] *S. typhimurium* G361 (*aroA*) harboring no plasmid or as indicated. Overnight cultures of G361 harboring pRSP-18 (2.1×10^8 CFU) were used for oral challenge. For G361 harboring pRIT10810, the challenge dosage was 5.8×10^8 CFU.

[b] Reciprocal of dilution giving OD = 1.0 in ELISA. Wells were coated with GM_1-ganglioside followed by cholera toxin. Mice were bled by the tail vein 12 days post-challenge. Results are the average of 5 wells.

Anti-CTB activity is additionally assessed for the ability to neutralize the biological activity of cholera toxin (CT). CTB-specific antibodies of various isotypes have been shown to neutralize the CT biological activity (Lycke *et al.*, 1985). Serum dilutions are incubated with various concentrations of CT at 25°C for 1 hr, with purified CTB-specific antibodies serving as controls. Neutralization of CT is assessed by inhibition of CHO cell elongation, (Guerrant *et al.*, 1974). Dilutions of commercial CT and CTB-specific antisera are used to standardize this assay.

To demonstrate that CTB on the surface of the *Salmonella* OM leads to the induction of protective immunity which abrogates CT activity *in vivo*, ligated ileal loops may be constructed. Many studies to define

the molecular mechanism(s) of action of CT have utilized this model. For these studies, groups of mice are challenged orally with: a) *Salmonella*/pRIT10810; b) *Salmonella*/pRSP-18; c) CTB (10-100 μg); d) PBS, or e) *Salmonella* alone. Effectiveness of the immunization is followed by determining serum titers to CTB. Ligated ileal loops (2 x 5 cm) are placed in immunized mice and challenged with various levels of purified CT (0-50 μg) for various times (6-24 hrs). Effectiveness of CT-neutralizing activity is assessed by measuring cAMP level sin intestinal tissue (Guerrant *et al.*, 1974) and measuring luminal fluid accumulating in the ligated loop at 6-24 hrs postchallenge. Protection by *Salmonella*/pRSP-18 shows that OM CTB expression is effective in producing protective immunity.

Mice challenged i.p. with purified CTB and a *Salmonella* strain expressing a CTB fusion protein are assessed for *in vitro* T cell activation. At ten and twenty days following i.p. challenge, purified splenic T cells are assessed for proliferation following *in vitro* stimulation with the following: a) *Salmonella* porin, b) whole cell lysates of the *Salmonella* CTB and CTB fusion expressing strains, c) whole cell lysates of *E. coli* D-12, d) CTB or e) PBS. Splenic T cells are assessed using microtiter cultures as previously described (Ramarathinan *et al.*, 1991). Peritoneal macrophages are used as antigen-presenting cells. In these studies, macrophages (2.5×10^4/well) are pulsed with different concentrations of test antigen for 2 hrs and washed before T cells (2×10^5/cells) are added to cultures. Cultures are assessed for proliferation at 2-4 days using [^{3}H]-thymidine incorporation.

EXAMPLE 10

This example illustrates insertion of a fragment of HIV Δgp160 gene into pZIP-OUT of Example 2.

CONSTRUCTION OF pZIP-OUT2 ENCODING A 60 kDa FRAGMENT OF HIV ΔGP160

The present invention also includes construction and characterization of a pZIP-OUT derivative which expresses HIV Δgp160 (gp120/gp41) epitopes in *Salmonella*. gp120 and gp41 are produced by the cleavage of the precursor gp160 molecule (Hu *et al.*, 1986). This gene was chosen because: 1) AIDS continues to be a major public health problem; 2) effective mucosal immunity may play a significant role in early host protection to HIV; 3) extensive information has accumulated as to the structure and function of this gene and its product. gp120 mediates HIV interaction with human CD4 cells (LaRosa *et al.*, 1990). In this regard, the predominant neutralizing determinant (PND) of HIV (LaRosa *et al.*, 1990) is located on the V3 loop of pg120; 4) antibodies to gp120 and gp41 are commonly observed in the sera of infected individuals; 5) mutation studies and competition studies with synthetic peptides (McPhee *et al.*, 1990) have shown that gp120 and gp41 interaction is required for viral infectivity. Thus, antibody binding to the PND or in the region of gp120-gp41 interaction will disrupt viral replication; and 6) purified recombinant gp120 and gp160 are currently under investigation as vaccine candidates.

A clone containing a 3.1 kb *Sal*I-*Xho*I fragment encoding the HIV gp160 gene was obtained from Dr. M. Cloyd (Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX). *Pvu*II digestion of this fragment yielded a 1.8 kb

fragment that deleted 0.7 kb of gp120 coding sequence. The 4.5 kb pZIP-OUT cassette, bounded by *Hin*dIII sites, was cloned into the *Hin*dIII site of vector pAT153 (Δ*Pvu*II site). This construction has been designated pZIP-OUT-2. pZIP-OUT-2 was digested with *Pvu*II and *Sal*I, and the *Puv*II - *Xho*I HIV fragment ligated into these sites. The tribrid fusion polypeptide from this construction yielded a 82 kDa polypeptide (2-4 kDa, *Salmonella*; 20 kDa, *pho*A; and 60 kDa, Δgp160).

Western analysis of outer membrane preparations of *Salmonella* harboring pAH13 was performed (FIG. 1). An immunoblot probed with an anti-gp120 polyclonal antibody showed a *Salmonella*::PhoA::Δgp160 fusion protein having a molecular mass of 84 kDa, the predicted value.

Both IgG and IgA antibody responses to gp120/gp41 may be determined from serum and extracts from intestinal scrapings. Antibodies to gp120/gp41 are detected by immunoblotting on separated viral proteins and level of antibodies determined by a quantitative HIV assay or a trans-activation assay. In this assay, pre-titered HIV stocks (treated and parallel untreated controls) are serially diluted in appropriate buffers and inoculated into individual wells of a 48-well tissue culture plate containing 10^5 $CD4^+$ cells suspended in 200 μL of culture medium containing 3 μg/mL polybrene. After 48 hours, an additional 100 μL of medium containing 30 μg dextran sulfate/mL is added to inhibit extracellular viral spread. Five days after inoculation, cells are assayed using a fixed-cell IFA with M26 monoclonal antibody (MoAb). The percentage of cells staining positive for viral core antigen p24, relative to cels infected with untreated HIV at a given dilution of virus reflects the decrease of inf ctivity in treated virus. Alternatively, dilutions of HIV may be inoculated into a $CD4^+$ cell line, H938, carrying an integrated copy of the HIV-1 long

terminal repeat (LTR) linked to the chloramphenicol acetyltransferase (CAT) gene (Felber and Paulakis, 1988). Thirty-six to 48 hours post-infection, cells are washed, lysed, and assayed for CAT activity using a commercially available kit. The highest dilution of virus showing enhanced CAT activity, when compared to cells infected with untreated HIV, allows quantitative assessment of the antiviral effect of a compound. Results from these studies reveal whether mucosal and/or systemic antibodies result from immunization of mice with a *Salmonella* surface-expressed Δgp160 fusion protein.

EXAMPLE 11

DNA SEQUENCING OF FUSION VECTORS

pZIP-IN, pZIP-OUT, and pRSP-18 were sequenced by the Sanger dideoxy protocol for double stranded DNA templates.

Preparation of Templates

Purified plasmid preps for sequencing were prepared as follows:

1. Each strain was grown overnight in 5 ml of LB broth (containing the appropriate antibiotic) at 37°C with vigorous aeration.
2. The cultures were harvested by centrifugation. The cell pellets were resuspended in 100 μl of 50 mM glucose, 10 mM EDTA, and 25 mM Tris-HCl, pH 8.0, and incubated at room temperature for 5 minutes.
3. 200 μl of freshly prepared .2N NaOH, 1% SDS were added to each sample. The samples were mixed by inversion, and then incubated 5 minutes on ice.

4. 50 µl of 3 M potassium acetate (pH 4.8) were added to each sample. The samples were mixed by inversion and incubated for 5 minutes on ice.
5. The samples were then centrifuged for 5 minutes, and the supernatants transferred to fresh tubes. The samples were centrifuged a second time for 5 minutes and the supernatants transferred as before.
6. RNase A was added to a concentration of 20 µg/ml, and the samples were incubated at 37°C for 20 minutes.
7. Each sample was phenol/chloroform extracted, chloroform extracted, and then ethanol-precipitated.
8. The DNA precipitates were collected by centrifugation and each DNA pellet was resuspended in 16 µl deionized water, 4 µl 4 M NaCl, and 20 µl 13% polyethylene glycol 8000. The samples were mixed well and incubated on ice for 20 minutes.
9. The samples were centrifuged 10 minutes and the supernatants discarded. The pellets were washed twice in 70% ethanol, dried, and resuspended in 20 µl of dH_2O.

Denaturation, Annealing, and Sequencing of Templates

For each DNA template prepared as above:

1. 2 µl of 2 M NaOH/2 mM EDTA were added to the entire 20 µl sample and the sample was incubated for 10 minutes at room temperature.
2. The reactions were neutralized by the addition of 4.5 µl of 2 M sodium acetate (pH 5.0) and 5.5 µl of dH_2O. The samples were mixed well, and then precipitated with 100% ethanol.

3. The DNA pellets were collected by centrifuging for 15 minutes. The pellets were then washed once with 70% ethanol and dried.

4. All of the following reagents, except primers and radioactive label, were supplied in the Sequenase™ sequencing kit (Amersham Corp., Arlington Heights, IL). The dried pellets were resuspended in 7 μl dH_2O, 2 μl of 5X Sequenase reaction buffer and 1 μl (~20 ng) of the appropriate primer. For sequencing the *Salmonella* sequences in pZIP-IN and pZIP-OUT, immediately upstream from the *phoA* junction, primer 1 was used. For extended sequencing in the *Salmonella* sequences of pZIP-OUT, primer 2 was utilized. To sequence across the *phoA:ctxB* junction in pRSP-18, primer 3 was used. The annealing reactions were incubated for 30 minutes at 37°C.

5. To each annealing mixture, 2 μl of .1M dithiothreitol, 2 μl of diluted labelling mix, 1 μl of [S^{35}]-dATP, and 2 μl of diluted Sequenase™ enzyme were added. The reactions were mixed and incubated at room temperature for 5 minutes.

6. 3.5 μl of each labelling reaction were then transferred to each termination mixture tube, containing dideoxy ATP, dideoxy GTP, dideoxy CTP, and dideoxy TTP. The chain termination reactions were allowed to proceed for 5 minutes at 37°C.

7. 4 μl of stop solution were added to each reaction, and the reactions were heated to 75°C for 2-5 minutes.

8. The reactions were loaded onto a 6% acrylamide-urea sequencing gel and electrophoresed at 15 mA for 2-6 hours.

9. After electrophoresis, the sequencing gel was fixed in 10% methanol, 10% acetic acid, for 1 hour and then dried under vacuum for 1 1/2 hours.

10. The dried gel was then exposed to autoradiograph film at room temperature for ~16 hours.

EXAMPLE 12

This example illustrates insertion of a fragment of influenza HA epitope into pZIP-OUT vector of Example 2.

CHARACTERIZATION OF PLASMIDS EXPRESSING INFLUENZA HEMAGGLUTININ EPITOPES AND THEIR USES FOR INFLUENZA VACCINES

The present invention describes the construction and characterization of export-specifying plasmids which express influenza strain A/PR/8/34 hemagglutinin (HA) epitopes in the surface of a virulence-attenuated *Salmonella* strain.
These strains were constructed using pZIP-OUT.

It is well established that HA of influenza is a critical viral structure for immune defense mechanisms (Stuart-Harris, 1979). Antibodies to HA are produced during natural infection (Schulman, 1975) and after vaccination (Schulman, 1975). A number of studies have shown that it is antibody to HA which blocks infection *in vitro* and *in vivo* (Stuart-Harris, 1979; Schulman, 1975). Thus, antibody to HA is a critical component in antiviral immunity to influenza virus.

HA mediates the absorption and penetration of influenza virus into susceptible cells, and hence, is

well established as an critical target for immune defense mechanisms (Wilson *et al.*, 1981; Stuart-Harris, 1979). In these studies, the consequence of immunization with *Salmonella*-expressed HA is investigated on the ability of mice to mount an effective host defense to influenza virus A/PR/8/34. At various times post-boost (10-21 days), groups of immunized mice are challenged intranasally with 3-30 LD50 or 1-10 MPD50 (mouse pneumonia dose) of influenza virus. The effectiveness of the various immunization protocols is evaluated by a comparison of survival in the lethality studies and a comparison of the pneumonic consolidation lesion scores (Gerone *et al.*, 1971). These results are evaluated in the context of the antibody titers and for significance using an appropriate statistical model.

The influenza HA1 gene chosen for these studies because: 1) it is readily available (American *Type Culture* Collection, 1990, #39736); 2) it is of optimal size for these genetic manipulations and has been completely sequenced (Caton *et al.*, 1982); 3) it contains immunodominant regions (HA1) which have previously been identified (Green *et al.*, 1982) and correlated with topographically distinct regions on the surface of the polypeptide (Wilson *et al.*, 1981); 4) it is well established that HA is a critical target structure for humoral immune defense (Stuart-Harris, 1979); 5) it is known that mucosal immunity is essential to effective host defense; and 6) an exquisitely sensitive, murine animal model is available to assess immunity to influenza virus (Gerone *et al.*, 1971). Thus, HA fusion polypeptides expressed on *Salmonella* vaccine strains used to orally immunize mice represent an ideal model. Such *Salmonella* strains have utility as influenza vaccine candidates. Advantages of this recombinant vaccine are increased stability, low cost, and elimination of costly purification protocols used with the current vaccine.

Generati n of the *Salmonella*::HA1 Gene Fusion

The cloned HA1 gene from influenza virus A/PR/8/34 was utilized. This gene and the parental virus from which it was derived are available from ATCC (American *Type Culture* Collection, 1990, #39736). Gene fusions into pZIP-OUT utilized methods analogous to those described hereinabove for *ctxB* (CTB). Specifically, HA1 DNA sequences were restricted with *Hpa*I at position 175 (Caton *et al.*, 1982), and blunt end ligated into the *Ssp*I or *Pvu*II site of pZIP-OUT. This joined the HA1 polypeptide to the *Salmonella* export sequences at residue 30 (Asn) and resulted in a fusion protein which retains 295 of the 325 HA1 amino acids (Caton *et al.*, 1982). This strategy insures the retention of the operationally distinct Sa, Sb, Ca, and Cb antigenic sites of HA (Caton *et al.*, 1982; Green *et al.*, 1982; Wilson *et al.*, 1981). The continuity of the reading frame across the fusion joints was confirmed by DNA sequencing.

Western Analysis of Resulting Fusion Protein

Expression of appropriate fusion polypeptides were evaluated by immunoblot analysis using HA-specific antibodies. Membrane expression of the fusion polypeptides was confirmed by observation of the predicted 43 kDa fusion polypeptide in isolated OM fractions (Fillip *et al.*, 1973). To determine if surface-expressed HA is biologically active on the *Salmonella* surface, *Salmonella* harboring the pZIP-OUT vector alone, the HA1-encoding pZIP-OUT derivative, and a control strain were characterized for hemoagglutinin or hemagglutination inhibition (Gerone *et al.*, 1971) activity.

EXAMPLE 13

This example illustrates insertion of a fragment of SFG rickettsial outer membrane p190 into the pZIP-OUT vector of Example 2.

CHARACTERIZATION OF PLASMIDS EXPRESSING SFG RICKETTSIAL OM p190 EPITOPES

In this example, the inventors contemplate the construction of a pZIP-OUT derivative which expresses SFG rickettsial outer membrane (OM) p190 (Anderson *et al.*, 1990) epitopes. A 1.87 kb *Nae*I-*Hpa*I fragment which contains the sequence representing the species-specific epitope (Anderson *et al.*, 1990) has been constructed. Retention of an open reading frame for the construct may be confirmed by DNA sequencing across the fusion joint. plasmids may then be introduced into *Salmonella* by electroporation. Expression of the predicted fusion polypeptides is evaluated by immunoblot analysis using SFG p190 sera. The membrane localization of the fusion protein is then determined by immunoblot analysis following fractionation of the *Salmonella* strains to yield OM, total envelope, and inner membrane fractions (Fillip *et al.*, 1973). Finally, surface preparations may be assessed for immunoreactive epitopes.

EXAMPLE 14

Several different types of heterologous proteins have been successfully directed to the outer membrane of *Salmonella*. The vector employed was pZIP-OUT. Included are an ~85 kDa tribrid fusion protein encoded by 1.8 kb HIV Δgp160 gene segment, ~52 kDa and ~68 kDa fusion polypeptides encoded by a 1.1 kb and a 1.6 kb gene segment of the influenza virus hemagglutinin gene and a

32 kDa CTB fusion protein. All polypeptides were found in OM fractions. These data are summarized in Table 3.

pZIP-OUT represents an export-directing vector that transports the products of diverse heterologous gene segments to the *Salmonella* OM.

EXAMPLE 15

To show that the plasmid-encoded *Salmonella* export sequences direct APase fusion proteins to the cell envelope, subcellular fractions of *Salmonella* strains harboring pZIP-IN or pZIP-OUT were prepared to yield total envelope (TE), OM and IM preparations. These were utilized for Western analysis and probed with a MoAb to APase. An APase fusion protein in pZIP-OUT-containing cells was observed at high levels in the OM and TE, while only trace amounts were present in the IM. For pZIP-IN containing cells, an APase fusion protein was observed in the IM and TE while only trace amounts were seen in the OM.

In other studies, surface-encoded polypeptides were extracted from *S. typhimurium* harboring either pZIP-IN or pZIP-OUT by mild urea extraction. In Western analysis of these surface extracts probed with a MoAb to APase, only pZIP-OUT showed surface expression of an APase fusion protein, indicating that APase is directed to the IM or OM by export-specifying motifs of pZIP-IN and pZIP-OUT, respectively.

TABLE 3

SUMMARY OF THE SUBCELLULAR LOCALIZATION OF FUSION POLYPEPTIDES EXPRESSED BY EXPORT-DIRECTING PLASMIDS

Plasmids[a]	Expressed fusion polypeptide[b]	Subcellular localization of fusion polypeptide in:[c]				
		CYT	TE	IM	OM	SURF
pZIP-IN	APase	-	+	+	-	-
pZIP-OUT	APase	-	+	-	+	+
pIMB13	Δ APase + CTB	-	+	+	-	-
pRSP-18	Δ APase + CTB	-	+	-	+	+
pBDHA1.6	Δ APase + HA	+	+	-	+	ND
pBDHA1.1	Δ APase + HA	+	+	-	+	ND
pAH13	Δ APase + Δgp160	+/-	+	-	+	ND

[a] pBDHA1.1 and pBDHA1.6 are derivatives of pZIP-OUT and express a 52 kDa and 68 kDa influenza virus hemagglutinin fusion protein, respectively, pAH13 is a derivative of pZIP-OUT which expresses a fusion protein which is ~85 kDa which contains ~20 kDa of HIV sequence, all of gp41 and export-specifying and PHOA sequences.

[b] Composition of hybrid polypeptides containing *Salmonella* export sequences. (Δ-deletion, AP-APase; HA-influenza virus hemagglutin; Δgp160-human immunodeficiency virus polyprotein with Δgp120 and gp41 sequences).

[c] CYT (cytosolic fraction) TE (total envelope). IM (inner membrane) and OM (outer membrane) were separated by sarkosyl extraction (Fuhrman and Cebra, 1981). SURF (surface extractions) were prepared by urea solubilization (Manoil and Beckwith, 1986). APase or CTB were detected by Western analysis. HA and Δgp160 fusion proteins were detected by immunoblotting using commercially obtained MoAb; Δgp160 MoAB (AMAC, Inc; Westbrook, ME); HA MoAB; (O.E.M. Concepts Inc., Boston, MA).

The ability of the pZIP-IN and pZIP-OUT to facilitate envelope expression of large segments of other genes (X) was also evaluated. These studies utilized sites within the phoA gene which maintain the correct reading frame to produce *Salmonella*:PHOA:X tribrid fusion proteins. Using this approach, the *V. cholera ctxB* gene that encodes CTB was introduced into restriction sites in the *phoA* gene of pZIP-IN and pZIP-OUT that maintained the reading frame from *phoA* into *ctxB*. Both constructs predict a 32 kDa tribrid fusion product containing *Salmonella*, *phoA* and virtually the entire sequence of CTB. Western analysis of whole cell extracts using a CTB-specific antisera showed that this fusion product was expressed. pRSP-18 and pIMB13 are derived from pZIP-OUT and pZIP-IN, respectively.

CTB fusion proteins were observed in association with IM and OM fractions of TA1972 harboring pIMB13 and pRSP-18, respectively. Purified OM from TA2362 containing pRSP-18 shows the presence of a 32kDa polypeptide that was recognized by antisera to CTB. IM from this strain showed no CTB cross-reactive polypeptide. However, in TA2362 harboring pIMB13, a cross-reactive 32kDa band with this same antisera was present only in TE and IM fractions, and not in OM fractions. In addition, only surface extracts from pRSP-18 harboring cells showed a 32 kDa-reactive band.

Further studies were conducted to determine whether or not the 32kDa surface-expressed CTB-fusion protein encoded by pRSP-18 was released into the medium during the growth of this strain. Concentrated culture supernatants from TA2362 harboring pRSP-18, pIMB13 or no plasmids did not show the presence of the 32 kDa fusion protein by immunoblotting. These data indicated that the 32kDa fusion proteins encoded by pRSP-18 and pIMB13 were not released in detectable quantities during *in vitro*

growth. In a single preliminary experiment, *Salmonella* strains harboring pRSP-18, pIMB13 or no plasmids were placed in ligated murine ileal loops for 8 hr. A 32 kDa polypeptide band was not observed in clarified intestinal fluid and/or washings from loops infected by any of the strains by immunoblot analysis.

CTB epitopes on live TA2362 harboring pRSP-18 were also demonstrated. Antisera to CTB agglutinated TA2362 pRSP-18, but not this strain alone or TA2362/pIMB13, indicating that CTB expressed by pRSP-18 is exposed on the surface of the cell and that the tribrid fusion protein encoded by pIMB13 is associated with the IM.

Whole cells and cultures supernatants were prepared from *S. typhimurium* TA2362 alone and TA2362 harboring pIMB13 and pRSP-18. Cells from overnight cultures (50 µl) and supernatants (concentrated 12.5-fold by ultrafiltration) were denatured in SDS-sample buffer, separated in denaturing 10% PAG and transferred to nitrocellulose. Blots were probed with affinity-purified CTB-specific antibodies (1/500) followed by goat anti-rabbit horseradish peroxidase-conjugated antibodies (1/4000) and bands visualized by fluorography using enhanced chemiluminescence (ECL, Amersham Corp., Arlington Heights, IL) development. Kodak XAR film was exposed for 45 sec. Lanes: A, TA2362, whole cell extracts (WC); B, TA2362, concentrated supernatants (CS); C, TA2362(pIMB13) WC extract; D, TA2362(pRSP-18) CS; E, TA2362(pIMB13) WC extract; F, TA2362(pIMB13) Cs. A CTB-reactive 32 kDa fusion protein is seen only in WC extracts (lanes C and E) and not in CS. The 32kDa fusion protein is shown with an arrow. A nonspecific protein band (~30 kDa) was observed in all WC extracts (lanes A, C and E) and is shown by the X.

EXAMPLE 16

The inventors contemplate the use of the present system in raising antibodies to peptides or polypeptides (Martineau *et al.*, 1991). These methods would be valuable in that the peptide or polypeptide would not need to be purified and screening of these antibodies may utilize the surface-expressed antigens.

GENERATING ANTIBODIES TO FUSION PEPTIDES OR POLYPEPTIDES AND THE DETECTION THEREOF

An antibody can be a polyclonal or a monoclonal antibody. In a preferred embodiment, an antibody is a monoclonal antibody. Means for preparing and characterizing antibodies are well known in the art (See, *e.g.*, *Antibodies "A Laboratory Manual,"* E. Howell and D. Lane, Cold Spring Harbor Laboratory, 1988).

Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen comprising a polypeptide of the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically an animal used for production of anti-antisera is a rabbit, a mouse, a rat, a hamster or a guinea pig. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

Antibodies, both polyclonal and monoclonal, specific for the surface-expressed polypeptides and/or proteins of the present invention may be prepared using conventional immunization techniques, as will be generally known to those of skill in the art. A composition containing antigenic epitopes of the surface-directed polypeptide can be used to immunize one or more experimental animals,

such as a rabbit or mouse, which will then proceed to produce specific antibodies against the surface-directed polypeptide. Polyclonal antisera may be obtained, after allowing time for antibody generation, simply by bleeding the animal and preparing serum samples from the whole blood.

To obtain monoclonal antibodies, one would also initially immunize an experimental animal, often preferably a mouse, with an antigenic composition. One would then, after a period of time sufficient to allow antibody generation, obtain a population of spleen or lymph cells from the animal. The spleen or lymph cells can then be fused with cell lines, such as human or mouse myeloma strains, to produce antibody-secreting hybridomas. These hybridomas may be isolated to obtain individual clones which can then be screened for production of antibody to the desired surface-directed polypeptide.

Following immunization, spleen cells are removed and fused, using a standard fusion protocol (see, *e.g.*, The Cold Spring Harbor Manual for Hybridoma Development, incorporated herein by reference) with plasmacytoma cells to produce hybridomas secreting monoclonal antibodies against the surface-directed polypeptide. Hybridomas which produce monoclonal antibodies to the selected antigens are identified using standard techniques, such as ELISA and Western blot methods.

Hybridoma clones can then be cultured in liquid media and the culture supernatants purified to provide the fusion protein-specific monoclonal antibodies.

It is proposed that the monoclonal antibodies of the present invention will find useful application in standard immunochemical procedures, such as ELISA and

Western blot methods, as well as other procedures which may utilize antibody specific to the fusion protein epitopes.

Antibodies generated against surface-directed polypeptides will also be useful in immunolocalization studies to analyze the distribution of fusion proteins on the bacterial surface. The operation of all such immunological techniques will be known to those of skill in the art in light of the present disclosure.

Surface-expressed polypeptides may also be used to detect antibodies. The bacterium would function as a solid phase support. This could have high utility for diagnostic kits to detect and diagnose infectious agents or other disease states (i.e. Grave's disease) which result in the production of specific antibodies. Alternatively, surface-expressed polypeptides or peptides may be used analogously to bacteriophage display libraries (Barbas *et al.*, 1991).

The compositions of the present invention will find great use in immunoblot or western blot analysis. Antibodies generated against the surface-expressed polypeptides may be used as high affinity primary reagents for the identification of proteins immobilized onto a solid support matrix, such as nitrocellulose, nylon or combinations thereof. In conjunction with immunoprecipitation, followed by gel electrophoresis, antibodies to the surface-expressed polypeptides may be used as a single step reagent for use in detecting antigens against which secondary reagents used in the detection of the antigen cause an adverse background. This is especially useful when the antigens studied are immunoglobulins (precluding the use of immunoglobulins binding bacterial cell wall components), the antigens studied cross-react with the detecting agent, or they

migrate at the same relative molecular weight as a cross-reacting signal.

It is also useful in the amplification of the signal or means of detection, by providing a means of amplifying the chromogenic signal, for example, by allowing for specific binding of numerous enzyme-conjugated anti-surfaced-expressed polypeptide antibodies to multiple fusion protein or native molecules attached to the antibody. This amplification is useful regardless of the method of visualization. Although not necessary to the routine practice of the present invention, it is contemplated that different detection techniques may be employed advantageously in the visualization of each of the antigens being detected. Immunologically-based detection methods for use in conjunction with western blotting include enzymatically-, radiolabel-, or fluorescently-tagged secondary antibodies against the toxin moiety are considered to be of particular use in this regard.

It is also envisioned that antibodies expressed on the surface of bacteria may be selected with appropriate antigens by bacterial display techniques (Fuchs *et al.*, 1991).

EXAMPLE 17

SURFACE EXPRESSION OF ENZYMATIC PROPERTIES OF FUSION PROTEINS

The inventors further contemplate the engineering of surface-expressed polypeptides to allow for surface-expression of their enzymatic properties. Bacteria harboring these constructs may be used as biocatalysts in applications in which the bacterial cell is used as a solid support (Kessler, 1981). This may lead to use in

diverse applications such as industrial processes to produce specific compounds to likely bioremediation applications. Other activities of the surface-expressed polypeptides could also be exploited. For example, polypeptides that bind specific molecules could be used to isolate or quantitate these molecules. In this regard, surface-expression of a receptor would allow convenient isolation of the molecule which is bound from diverse or complex sources such as bodily fluids or from the environment. An analogous, albeit more limited system which is currently used, is the protein A molecule of *Staphylococcus aureus* that functions as a receptor for immunoglobulins (Ig) and is useful in their quantitation and purification.

EXAMPLE 18

THE UTILITY OF SURFACE-EXPRESSION FUSION PROTEINS IN IMMUNOAFFINITY CHROMATOGRAPHY

The fact that *Salmonella* peptide sequences are present at the N-termini of surface-expressed fusion proteins generated by the methods described herein also suggests the utility of the fusion proteins of the present invention in efficiently purifying polypeptides by immunoaffinity chromatography. Antibodies directed to the *Salmonella* sequences immobilized on a column could be used in a general protocol to purify a wide range of fusion proteins which harbor this *Salmonella* export-directing sequence. These methods would be of high significance for low abundance proteins. The methods for immunoaffinity chromatography are well known to those skilled in the art (Harlow and Lane, 1988).

Affinity chromatography is generally based on the recognition of a protein by a substance such as a ligand or an antibody. The column material may be synthesized

by covalently coupling a binding molecule, such as an activated dye, for example to an insoluble matrix. The column material is then allowed to adsorb the desired substance from solution. Next, the conditions are changed to those under which binding does not occur and the substrate is eluted. The requirements for successful affinity chromatography are that the matrix must adsorb molecules, the ligand must be coupled without altering its binding activity, a ligand must be chosen whose binding is sufficiently tight, and it must be possible to elute the substance without destroying it.

* * *

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are

deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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The references listed below are incorporated herein by reference to the extent that they supplement, explain, provide a background for or teach methodology, techniques and/or compositions employed herein.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) INVENTORS: NIESEL, David W.
MONCRIEF, J. Scott
PHILLIPS, Linda H.

(iii) TITLE OF INVENTION: MEMBRANE EXPRESSION OF HETEROLOGOUS GENES

(iv) NUMBER OF SEQUENCES: 2

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(F) ZIP: 77210

(vi) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS, ASCII
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vii) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: Unknown

(B) FILING DATE: Concurrently herewith

(C) CLASSIFICATION: Unknown

(viii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: SN 08/326,772

(B) FILING DATE: 18 OCTOBER 1994

(C) CLASSIFICATION: 1804

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(A) TELEPHONE: 512-418-3000

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(C) TELEX: 79-0924

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 213 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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          15                  20                  25

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Gly Cys Ala Pro Asp Ser Tyr Thr Gln Val Ala Ser Trp Thr
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TTTGTAGGTA TGGGGCTCAT AGCTGACTCT TATACACAAG ATGCGCCTGT 350

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CLAIMS:

1. An *in vivo* method of inducing antigen-specific antibodies comprising oral administration of *Salmonella typhimurium* or *E. coli* transformed with DNA encoding the antigen and a surface exportation DNA and allowing sufficient time for development of an immune response to the antigen.

2. The method of claim 1 wherein the surface exportation DNA is SEQ ID NO:1 or a sequence substantially homologous to SEQ ID NO:1.

3. The method of claim 1 wherein the antigen is cholera toxin B subunit.

4. The method of claim 1 wherein the antigen is influenza hemagglutin or influenza neuraminidase.

5. The method of claim 1 wherein the antigen is a human immunodeficency virus Δgp160, gp120, gp41, or gp24.

6. The method of claim 1 wherein the antigen is a 52 kDa or 68 kDa influenza virus hemagglutinin, a 62 kDa human immunodeficiency virus gp120/gp41, or a 32 kDa CtxB fusion protein.

7. A method of transporting a protein to the outer surface membrane of a bacterial cell, comprising:

fusing a DNA encoding cholera toxin B subunit, influenza hemagglutinin, influenza neuraminidase, human immunodeficiency virus Δgp160, gp120, gp41, or gp24, or rickettsial outer membrane p190 protein with DNA encoding a surface exportation protein;

transforming an *E. coli* or *Salmonella* with the fused DNA; and

culturing the *E. coli* or *Salmonella* to express said protein at or within the outer membrane of said bacterial cell.

8. A recombinant DNA comprising DNA that is identical to or homologous with the DNA of SEQ ID NO:1 fused with a DNA encoding an immunogenic protein.

9. A recombinant DNA comprising DNA that is identical or homologous with the DNA of SEQ ID NO:2 fused with a DNA encoding an immunogenic protein.

10. The recombinant DNA of claim 8 or claim 9 wherein the immunogenic protein is a cholera toxin B subunit.

11. The recombinant DNA of claim 8 or claim 9 wherein the immunogenic protein is a human immunodeficiency virus Δgp160, gp120, gp41, or gp24 protein.

12. The recombinant DNA of claim 8 or claim 9 wherein the immunogenic protein is influenza hemagglutinin or influenza neuraminidase.

13. The recombinant DNA of claim 8 or 9 wherein the immunogenic protein is a 52 kDa or 68 kDa influenza virus hemagglutinin, 62 kDa human immunodeficiency virus gp120/gp41 or 32 kDa CtxB fusion protein.

14. A pharmaceutical composition comprising *Salmonella* or *E. coli* transformed with the recombinant DNA of claim 8 or claim 9 in a pharmaceutically acceptable vehicle.

15. The pharmaceutical composition of claim 14 wherein expressed immunogenic protein is associated with the outer membrane surface of the *Salmonella* or *E. coli*.

16. The pharmaceutical composition of claim 14 wherein expressed immunogenic protein is associated with the periplasm of the *Salmonella or E. coli*.

17. The pharmaceutical composition of claim 15 or claim 16 wherein the immunogenic protein is cholera toxin B subunit, influenza hemagglutinin, influenza neuraminidase, HIV Δgp160, gp120, gp41, or gp24 protein, or rickettsial outer membrane p190 protein.

18. A method of *in vivo* induction of cholera toxin B subunit protective antibodies comprising:

orally administering to an animal a recombinant *Salmonella* or *E. coli* that periplasmically or outer membrane expresses cholera toxin B subunit wherein cholera toxin B subunit specific antibodies are produced in response to the administration.

19. The method of claim 18 wherein the recombinant *Salmonella* or *E. coli* harbors DNA that includes DNA encoding cholera toxin subunit B fused with the DNA of SEQ ID NO:1 or SEQ ID NO:2 or substantially homologous DNA sequence.

20. The method of claim 1 wherein the antigen is rickettsial outer membrane p190.

21. The recombinant DNA of claim 8 or claim 9 wherein the immunogenic protein is a rickettsial outer membrane p190 protein.

22. A pharmaceutical composition comprising outer membrane fragments of *Salmonella* or *E. coli* transformed with the recombinant DNA of claim 8 or claim 9 in a pharmaceutically acceptable vehicle, wherein said fragments have an immunogenic protein associated therewith.

23. The pharmaceutical composition of claim 22 wherein said immunogenic protein is cholera toxin B subunit, influenza hemagglutinin, influenza neuraminidase, human

immunodeficiency virus Δgp160, gp120, gp41, or gp24 protein, or rickettsial outer membrane p190 protein.

24. The pharmaceutical composition of claim 22 wherein expressed immunogenic protein is located in the periplasm of the *Salmonella or E. coli*.

25. A method of *in vivo* induction of rickettsial outer membrane p190 protective antibodies comprising:

orally administering to an animal a recombinant *Salmonella* or *E. coli* that periplasmically or outer membrane expresses rickettsial outer membrane p190 antigen wherein rickettsial outer membrane p190-specific antibodies are produced in response to the administration.

26. The method of claim 25 wherein the recombinant *Salmonella* or *E. coli* harbors DNA that includes DNA encoding rickettsial outer membrane p190 fused with the DNA of SEQ ID NO:1 or SEQ ID NO:2 or substantially homologous DNA sequence.

27. A method of *in vivo* induction of influenza hemagglutinin protective antibodies comprising:

orally administering to an animal a recombinant *Salmonella* or *E. coli* that periplasmically or outer membrane expresses influenza hemagglutinin antigen wherein influenza hemagglutinin-specific antibodies are produced in response to the administration.

28. The method of claim 27 wherein the recombinant *Salmonella* or *E. coli* harbors DNA that includes DNA encoding influenza hemagglutinin fused with the DNA of SEQ ID NO:1 or SEQ ID NO:2 or substantially homologous DNA sequence.

29. A method of *in vivo* induction of influenza neuraminidase protective antibodies comprising:

orally administering to an animal a recombinant *Salmonella* or *E. coli* that periplasmically or outer membrane expresses influenza neuraminidase antigen wherein influenza neuraminidase-specific antibodies are produced in response to the administration.

30. The method of claim 30 wherein the recombinant *Salmonella* or *E. coli* harbors DNA that includes DNA encoding influenza neuraminidase fused with the DNA of SEQ ID NO:1 or SEQ ID NO:2 or substantially homologous DNA sequence.

31. A method of *in vivo* induction of human immunodeficiency virus Δgp160, gp120, gp41, or gp24 protective antibodies comprising:

orally administering to an animal a recombinant *Salmonella* or *E. coli* that periplasmically or outer membrane expresses HIV Δgp160, gp120, gp41, or gp24 antigen wherein HIV-specific antibodies are produced in response to the administration.

32. The method of claim 31 wherein the recombinant *Salmonella* or *E. coli* harbors DNA that includes DNA encoding human immunodeficiency virus Δgp160, gp120, gp41, or gp24 protein fused with the DNA of SEQ ID NO:1 or SEQ ID NO:2 or substantially homologous DNA sequence.

33. The pharmaceutical composition of claim 14 or claim 22 further comprising an adjuvant.

34. A kit comprising in packaged combination:

a carrier means adapted to receive a plurality of container means in close confinement therewith;

a first container means including an attenuated *Salmonella* or *E. coli* transformed with the DNA of SEQ ID NO:1 or SEQ ID NO:2 fused with an immunogenic protein; and

a second container means including a pharmaceutically acceptable vehicle for admixing with said transformed *Salmonella* or *E. coli*.

35. A kit comprising in packaged combination:

a carrier means adapted to receive a plurality of container means in close confinement therewith;

a first container means including outer membrane fragments of *Salmonella* or *E. coli* transformed with the DNA of SEQ ID NO: 1 or SEQ ID NO:2 fused with an immunogenic protein; and

a second container means including a pharmaceutically acceptable vehicle for admixing with said membrane fragments.

36. The kit of claim 34 or 35 wherein the immunogenic protein is cholera toxin B subunit, human immunodeficiency virus Δgp160, human immunodeficiency virus gp120, human immunodeficiency virus gp41, human immunodeficiency virus gp24 protein, influenza hemagglutinin, influenza neuraminidase, or rickettsial outer membrane p190 protein.

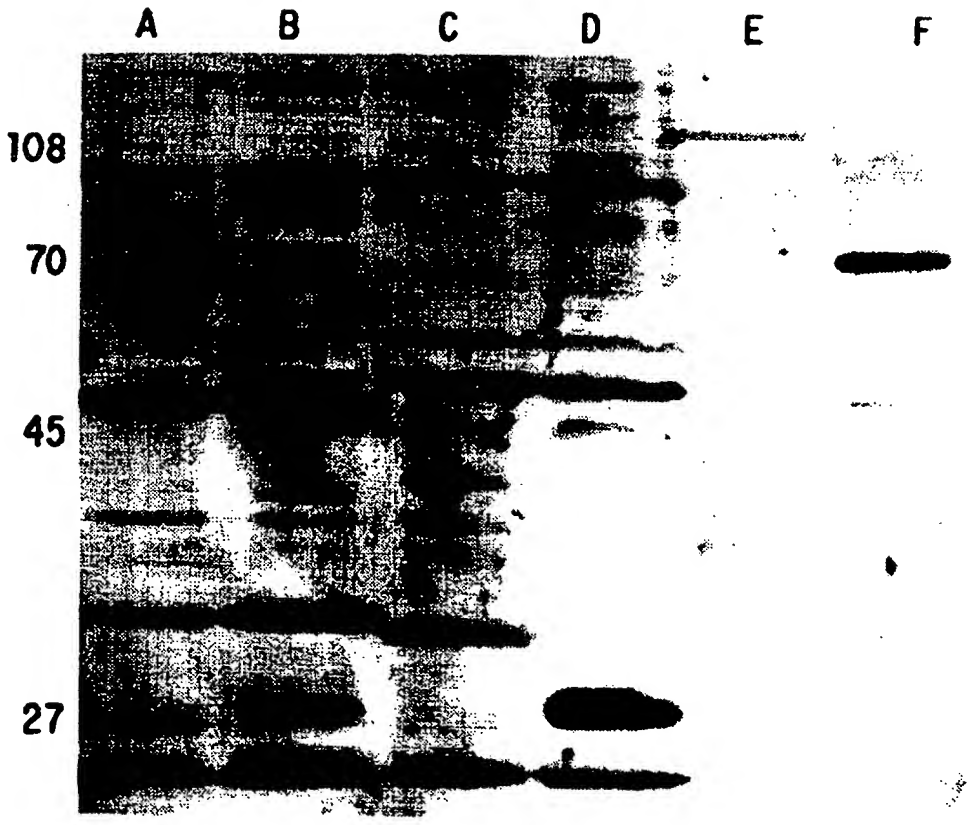

FIG. 1

FIG. 2

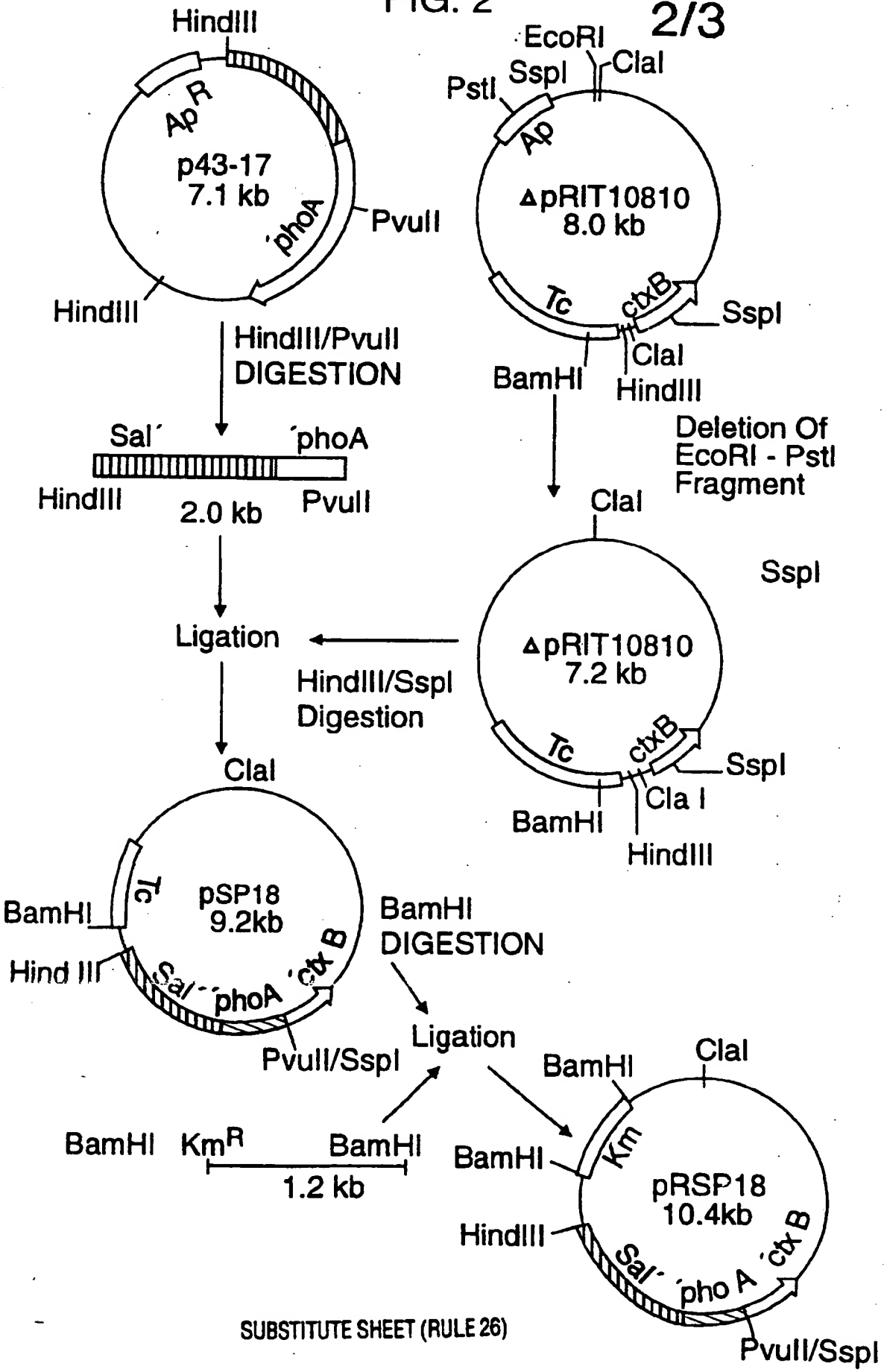
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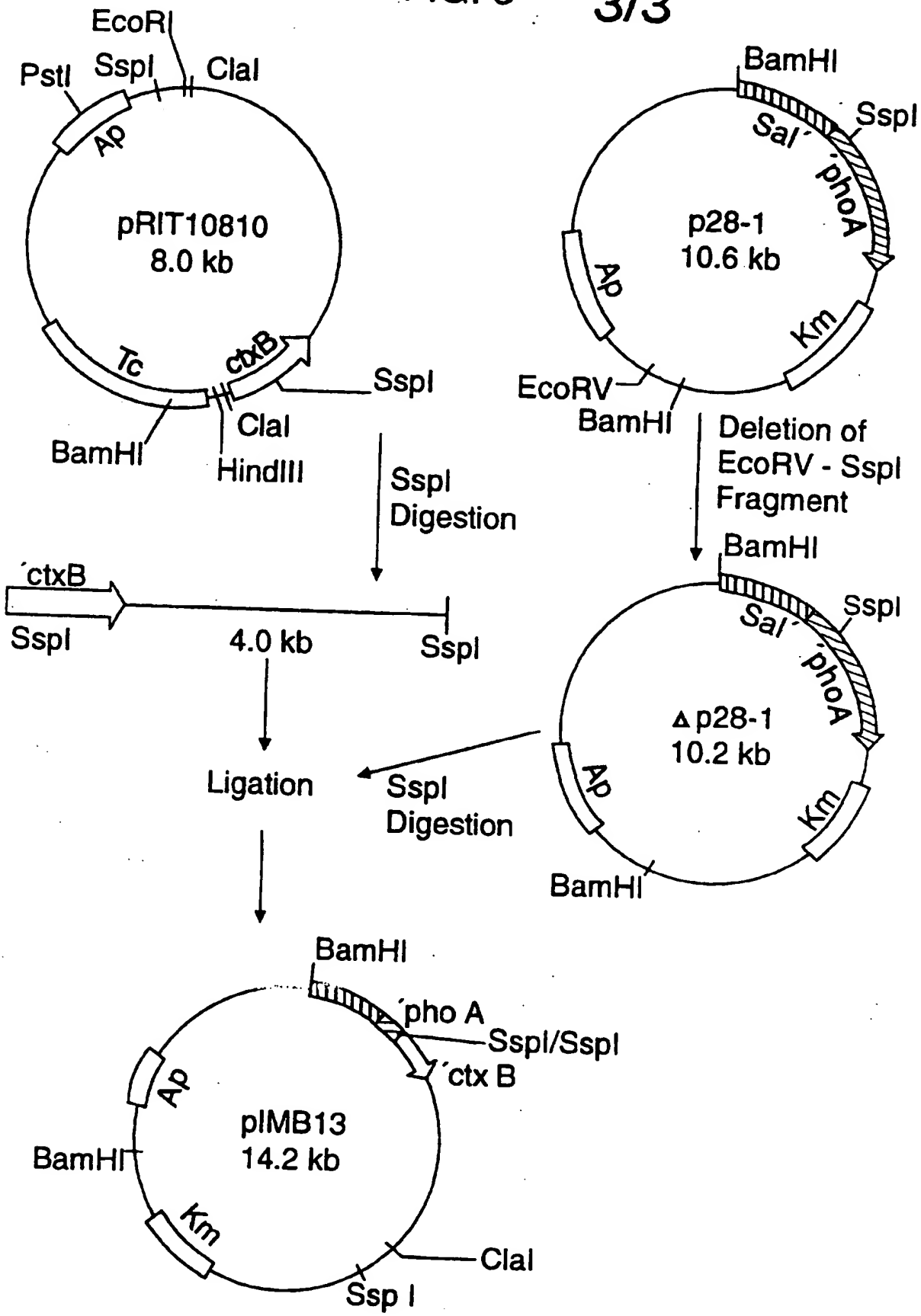
FIG. 3 3/3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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/13333

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.1, 184.1, 186.1, 190.1, 207.1, 208.1; 435/69.1,69.7; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Medline

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	WO 93/10246 (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM) see entire document, especially pages 5, 8, 9, 14, 15, 16, 34, 39, 40, 42.	1-3, 5-11, 13-19, 22-24, 31-36 --------- 4, 12, 20, 21, 25-30
X	US A, 5, 098, 998 (MEKALONAS ET AL) 24 March 1992, see columns 3, 9, 10.	14-18, 22-24, 33

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 09 FEBRUARY 1996	Date of mailing of the international search report 16 FEB 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer H. F. Sidberry Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/13333

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application N .
PCT/US95/13333

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 39/12, 39/106; C12N/15/62, 9/16, 15/31, 1/21; C07H 21/04; C12P 21/04

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/93.1, 184.1, 186.1, 190.1, 207.1, 208.1; 435/69.1,69.7; 536/23.1

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-7, 14-21, 25-33, drawn to in vivo method of inducing antigen-specific antibodies by giving Salmonella or E. coli transformed with DNA encoding antigens: cholera toxin B subunit; influenza hemagglutin or influenza neuraminidase; HIV; rickettsial outer membrane p190 and a surface exportation DNA, method of exporting a protein to the bacterial membrane surface classified in Classes 424 and 435 subclasses 93.2 and 93.1, 192.1, 197.1, 200.1 and 69.3, 69.7
Group II, claim(s) 8-13, drawn to recombinant DNA fused to DNA encoding an immunogenic protein, classified in Class 536 subclass 23.4.
Group III, claim(s) 22-24, 35, 36, drawn to pharmaceutical compositions comprised of membrane fragments of Salmonella or E. coli transformed with the DNA encoding the exportation protein associated with an antigen, classified in Class 424, subclasses 184.1, 186.1, 188.1, 190.1, 210.1, 207.1, 208.1
This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In orderformorethan one speciestobeexamined,theappropriateadditionalexamination fees must be paid. The species are as follows:
cholera toxin subunit B (claims 3, 6, 7, 10, 13, 17, 18, 19, 23, 24, 36); influenzae hemagglutin or neuraminidase (claims 4, 6, 7, 12, 17, 23, 27, 28, 29, 30, 36); HIV antigens(claims 5, 7, 11, 17, 23, 31, 32, 36); rickettsial outer membrane protein (claims 7, 17, 20, 21, 23, 25, 26, 36).

The following claims are generic: claims 1, 2, 8, 9, 14, 15, 16, 22, 24, 33, 34, 35.

The inventions listed as Groups I, II and III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The claims of these three groups are directed to inventions which are linked by a special technical feature. However, the technical feature which links the inventions is known in the art as exemplified the US Patent 5 098 998, which discloses using an exportation protein to express cholera toxin.
The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: the exportation protein is usedwithspecies of: cholera toxin subunit B; influenzae hemagglutin or neuraminidase; HIV antigens and rickettsial outer membrane protein which are considered physicochemically, structurally, antigenically and functionally distinct.